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# THE JOURNAL OF NUTRITION

## **JOHN R. MURLIN HONOR VOLUME**

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*John Raymond Murlin*

## JOHN R. MURLIN HONOR VOLUME

### VOLUME THIRTY-ONE

Through the special efforts of Dr. John R. Murlin, The Journal of Nutrition and a sponsoring organization, The American Institute of Nutrition, Inc., were established in 1928. In this venture Dr. Murlin had the assistance of ten leaders in the field of nutrition who served as members of an editorial board, namely, Eugene F. DuBois, Herbert M. Evans, Ernest B. Forbes, Graham Lusk, Elmer V. McCollum, Lafayette B. Mendel, Harold H. Mitchell, Mary S. Rose, Henry C. Sherman and Harry Steenbock. Arrangements were made for Charles C. Thomas of Springfield, Illinois, to serve as the publisher. The new Journal grew steadily in its acceptance among scientific workers and in its circulation. With respect to the latter, however, the growth was not sufficient to meet the deficits faced by the publisher. Dr. Murlin finally arranged for The Wistar Institute of Anatomy and Biology in Philadelphia to undertake its publication. This move on the one hand placed the resources of The Wistar Institute and its printing facilities behind the Journal, and on the other hand gave to The Wistar Institute and its long list of distinguished scientific periodicals representation in the field of nutrition. The original sponsoring organization, The American Institute of Nutrition, Inc., was also changed to a scientific society whose members are active investigators in the field of nutrition. This organization was eventually admitted to membership in the Federation of American Societies of Experimental Biology.

In 1939, when he had reached the age set for emeritus membership in the society, Dr. Murlin resigned his editorship. He continued at his post as Professor in the University

of Rochester until 1944 when he reached the retirement age of 70 years. Because the war had taken away so many of the younger members of the staff, Dr. Murlin was asked by his University to continue his services for a period which finally ended in June, 1945.

It is fitting that this Journal honor Dr. Murlin for the part he played in establishing it and in guiding it through its early years. This recognition takes the form of designating volume thirty-one, which begins with this issue, as the *John R. Murlin Honor Volume*. An article of appreciation written by one of his younger colleagues accompanies the photograph which serves as a frontispiece for this volume.

G. R. C.

## JOHN RAYMOND MURLIN

INVESTIGATOR, TEACHER, COLLEAGUE

### *An Appreciation*

It is well known that the experiences of childhood have much to do in shaping the character of a man. There are reasons for believing this to have been the case with the lad who was destined to become known as John R. Murlin, distinguished American investigator and teacher of physiology and nutrition.

In his early days in Ohio, young Murlin was occupied as assistant to his father, who operated a village store as well as a nearby farm. They were busy days filled with the marvelous variety of tasks and interesting adventures known only to the boy who grows up in the country. The environment of his youth accounts, in no small way, for the fact that he has always "kept his feet on the ground." After completing a normal school course he became a country school-master for a time in order to earn enough money to pursue his formal education further. He was awarded the B. S. degree in 1897 and the M. A. 2 years later by Ohio Wesleyan University where his major interests were biology and chemistry. Professor Conklin, the eminent zoologist from the University of Pennsylvania, on a visit to Ohio Wesleyan, met the enthusiastic young biologist who was then an instructor in charge of two courses in physiology. The result of this meeting was the offer of a fellowship in Conklin's department to the young man who was so eager to extend his scientific horizons and in 1901 Pennsylvania conferred on him the Ph. D. degree. His thesis was on digestion and absorption in the land isopods, indicating a manifest predilection for the functional rather than the structural aspects of his subject.

As a college student John Murlin met and fell in love with Josephine Seaman and they were married in 1899 while he was still a graduate student in Philadelphia. "Now it can be told" that she was taking a course in physiology at Ohio Wesleyan which, on account of the sudden illness of the regular instructor, her future husband was asked to teach, and that she withdrew immediately when she realized the embarrassment which her presence elicited in the substitute instructor. This discerning solicitude for the man of her choice has continued and grown for nearly half a century.

John Murlin was appointed Professor of Biology and Instructor in Chemistry at Ursinus College, a position which he held until 1903 when the opportunity of a lifetime presented itself in the form of an instructorship in the laboratory of the great Graham Lusk.

The new instructor came to Lusk's laboratory with definite ideas concerning his own research program — he wished to study the metabolism of proteins. Lusk, trained in the best German tradition, must have been somewhat taken aback by the confidence of the relatively inexperienced newcomer who insisted on independent investigation, but the Professor was persuaded and suggested a study of the nutritive value of gelatin which at that time was being much discussed as a substitute for meat. While engaged on this project, Murlin found time to translate, from the German, Tigerstedt's "Textbook of Human Physiology." Following the work on gelatin came a beautifully planned and executed investigation on the nitrogen and energy metabolism of pregnancy in dogs at Cornell, and in humans at the Nutrition Laboratory in Boston with Thorne Carpenter. Later more work appeared on human pregnancy and the metabolism of infants in collaboration with H. C. Bailey and with B. R. Hoobler. This work brought invitations to write the chapter on metabolism in Abt's "Pediatrics," and to deliver the Harvey Society lecture in 1917.

Ever since von Mering and Minkowski demonstrated pancreatic diabetes in 1889 many investigators had been in-

trigued by its similarity to diabetes mellitus in man and the possibility of discovering a therapy adequate for its control. Murlin, too, was attracted to this problem and proposed his scheme of attack to Professor Lusk, whose comment was, "Oh, but Minkowski tried that and failed." The work was undertaken, nevertheless, and in 1913 Murlin and Kramer published strong evidence that their extracts of pancreas were active. The diabetic dog, in many instances, responded to extract injection with increased R. Q. and diminished glycosuria and indeed in one experiment the urine remained free of reducing substances for 4 hours. In control experiments it appeared that the weak alkali, used to neutralize some of the extracts prior to administration, on injection gave results similar to those obtained with the extract itself. Much time was spent seeking an explanation of this unlooked for complication and the work was interrupted by our entry into World War I.

John Murlin was by strong conviction and family tradition impelled to take an active part in the war and, on leave of absence from Cornell, volunteered for officer training in the summer of 1917. His observations on the Army ration at Plattsburg training camp, with his practical suggestions for its improvement, soon brought him to the Surgeon General's Office in Washington as a major in the Sanitary Corps and an assignment to organize the Nutrition Division. This was accomplished with characteristic vigor and dispatch and for the first time the Army learned the actual food consumption of trainees under various conditions as well as the extent of food wastage. The data collected by Major Murlin, and some three score officers whom he brought into his organization, pointed the way to improvement of the ration on a nutritional basis rather than the traditional method of merely "filling the cavity" when a soldier got hungry. The overall result of this pioneer work was to awaken in the Quartermaster Department a broadened interest in its responsibility for feeding the soldier and in the Medical Department a new conception of its responsibility for his nutritional status. The

success of the Nutrition Division in the Army, where rights and responsibilities are rigidly prescribed by regulation and any threat to usurpation of prerogative is quickly eliminated, testifies to the versatility and adroitness of its director. He was promoted to a Lieutenant Colonelcy and finally separated from the service in the Spring of 1919.

Lewis P. Ross, former president of the board of trustees of the University of Rochester, bequeathed the greater portion of his estate to endow, " . . . a Department of Vital Economics which shall conduct instruction and experimentation in physiology, hygiene and nutrition of the human body to the end that human life may be prolonged with increased health and happiness." In 1916 John Murlin was invited to organize and direct the newly endowed department but declined because the university authorities were unsympathetic toward the newfangled idea of research. A year later, while he was still an officer candidate at Plattsburg, the offer was renewed and accepted with a clear understanding that the " . . . experimentation . . . " provided for in the terms of the bequest should receive its full share of attention. The University granted him leave of absence for the duration of the war, and he went off to the Surgeon General's Office in Washington. Despite his heavy responsibilities in the Army, the new director of the laboratory was able, by occasional hurried trips between Washington and Rochester, to plan and supervise an investigation on the antiscorbutic potency of dehydrated fruits and vegetables.

On completion of his Army service, he returned to Rochester to take active charge as Director of the Department of Vital Economics and Professor of Physiology. The staff was increased and graduate students began to arrive. Naturally, work was resumed on some of the prewar projects, including pancreatic diabetes, the metabolism of children and protein metabolism. The anti-diabetic substance was destined to be unequivocally demonstrated in another laboratory and Professor Murlin, concealing any personal regret he may have felt at having been unsuccessful in doing

the crucial experiments, joined scientific workers everywhere in acclaiming the magnificent achievement of Banting and Best. Their announcement was a stimulus to greater activity in Rochester and many papers appeared on the preparation, physiological effects and chemical properties of insulin.

In the quarter century since Professor Murlin took active charge, the Department of Vital Economics has accomplished a prodigious amount of work. Limitations of space do not permit even a listing of the papers that have been published, 220 odd, much less comments on them and the names of all the people who worked in the laboratory. The work in endocrinology broadened to include hormones of the thyroid, pituitary, adrenals, gonads and digestive tract. In a study of gluconeogenesis from fat a new calorimeter was devised which gave better agreement between direct and indirect heat measurements in man than any other apparatus hitherto described. The interest in proteins was intensified and extended and many papers were published on biological values as well as the protein sparing effect of various carbohydrates. The culmination of a long and active interest in protein metabolism came, appropriately, in the year preceding Professor Murlin's retirement when he was able with human subjects to characterize the biological values of certain proteins in terms of their constituent essential amino acids. A great deal of the work was done using human subjects and is a significant contribution not only in the placing of proteins in nutritional perspective but in the technique of nutritional investigations in man.

The immediate problems of the laboratory were not the only concern of its director. From 1919 to 1922 he was chairman of the Committee on Food and Nutrition of the National Research Council and again served as a member from 1941 through the greater part of World War II. In 1932 he was a member of the White House Conference on Child Health and Development and a delegate to the international conference on nutrition in Berlin sponsored by the Health Division of the League of Nations. He early saw the need for a journal



devoted especially to the publication of fundamental investigations in nutrition, and was the prime mover in the establishment of The Journal of Nutrition, which he edited through its first seventeen volumes (1928-1939); he had a most prominent part in organizing the Institute of Nutrition and is the only one of its members that has served two consecutive terms as president. The American Philosophical Society elected him to membership in 1932.

As a teacher of graduate students, Professor Murlin was firm in his insistence on a thorough grounding in the history and principles of his subject. He took great pains to present in seminars and lectures the development of the science of nutrition. When students reported on the classical works of Lavoisier, Liebig, Voit, Rubner and Lusk the Professor always listened eagerly to learn whether the neophyte had grasped the significant points and he was always ready to steer the discussion in order to correct misinterpretations and supply omissions. This he did easily and entertainingly because he was conversant not only with the details of the paper under discussion but often illuminating personal characteristics or incidents concerning its author. The historical aspect of science in general was pursued further at monthly Sunday evening meetings in the Murlin home. Here the whole department, wives included, enjoyed a buffet supper and social hour before the evening's reading was begun by some member of the department. These delightful intellectual excursions took one into the realms of chemistry, physics, medicine and even mathematics and astronomy. Mrs. Murlin, besides providing for the immediate nutritional needs of the "family", always took an active part in the reading and discussion. The memories of these gatherings in the gracious and cultured home of the Murlins are cherished by all who were privileged to participate.

In the laboratory there was great freedom of thought and action, for all who deserved it. Professor Murlin was vehemently opposed to pouring all students into the same mold. From the beginning of his own career with Lusk, he insisted

on independent investigation for himself and, after he became director of the laboratory at Rochester, he consistently encouraged students to embark on independent investigations as soon as they were ready for such ventures. As a logical consequence of this policy he refrained from attaching his name to every paper that came out of the laboratory just because he happened to be the director. He was exceptionally generous in this regard and shared the work as well as the credit in any research the results of which were published under joint authorship. Each new candidate began his post-doctorate career as the sole author of at least one paper. This represents a definite break in the traditional method of graduate instruction largely inherited from the European, and especially the German universities where the professor was often an autocrat, allegedly omniscient, who dominated a student's thinking and dictated his every move. It is a paradox still, in some American universities, that preaching the democratic ideal of freedom far outruns its practice.

The early recognition and encouragement of talent in investigation was considered fundamental. Students were urged to present results of their work before local and national meetings, and in the post-doctorate period, as soon as subsequent work confirmed the Professor's estimate of a man's capacity for productive scholarship, he was strongly supported in his application for membership in the appropriate professional society. Without being uncritical, Professor Murlin held no brief for exclusiveness in the fraternity of science.

Great devotion to the ideas and ideals of science is not always easy. One of his colleagues has said that among Professor Murlin's many achievements one of the most noteworthy was the initiation and courageous maintenance of a research program in an environment that at best was indifferent and often was downright hostile. How different the situation is a quarter of a century later! With the opening of the School of Medicine in 1925 and the advent of its staff of young and eager investigators it became much easier and even

fashionable to indulge in research. At present it is exceptional for any staff member not to be engaged in research and Professor Murlin stands in the van of the pioneers who brought about this complete change of attitude.

His exuberant initiative and vigorous activity were scarcely contained in over 4 decades of scientific endeavor; they overflowed into his recreation. The grounds at the Murlin home with their expanse of lawn, lovely trees and beautiful gardens give eloquent testimony to the inspiration and expert care of a master gardener. Students and colleagues frequently were invited to spend an afternoon at tennis, baseball, horseshoes or just relaxing in the shade waiting for supper al fresco. The Murlins are very fond of travel and have managed to see most of the United States and large portions of Europe, Canada and Mexico. When color film became available, Professor Murlin brought home most fascinating motion pictures of far places and peoples which, with Mrs. Murlin's lovely water colors, illustrated many an interesting travelogue of a Sunday evening. The Professor liked to motor to scientific meetings and the car was always filled with colleagues or students from the laboratory. His keen delight in exploring new places and his extensive knowledge of the flora, fauna and history of the regions visited made these trips rare occasions of high adventure.

On June 30, 1945, at age 71, John Murlin retired as Lewis P. Ross Professor of Physiology and Director of the Department of Vital Economics. He had generously agreed to defer his retirement a year on account of the depletion of the staff occasioned by the war. In his final year he probably worked harder than at any other time in his career at Rochester. He was an indefatigable worker up to the last day of his active duty and will doubtless continue in the same manner in whatever he chooses to do in the future. His constant vigorous attack on current problems was always a challenge to students and colleagues. Few achieved his pace but all were better for the attempt.

E. S. N.

# THE DETERMINATION OF METABOLIC FECAL NITROGEN AND PROTEIN DIGESTIBILITY

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## TWO FIGURES

(Received for publication August 9, 1945)

During the course of an investigation of the nutritive quality of proteins for growth (Bosshardt et al., '45) it was necessary to determine the "true digestibilities" of the proteins studied. This involved the estimation of the fecal nitrogen of non-dietary origin (metabolic fecal nitrogen) during the period of active growth.

At present, two general methods are available for the determination of the metabolic fecal nitrogen. The determination of the fecal nitrogen excretion of animals fed a nitrogen-free diet can be determined directly and has been used by some workers (Mitchell and Carman, '24) as a metabolic fecal nitrogen value. The inability of animals to grow and, with the smaller species such as the rat and the mouse, to maintain a normal food consumption on a nitrogen-free ration makes this method of limited use. Mitchell and Carman ('24) have indicated that whole egg protein is completely digestible. It has since become a practice of some investigators to incorporate a small amount of whole egg protein in the diet, usually 4 to 5%. The total fecal nitrogen excreted, assuming complete digestion of the whole egg protein, then would represent the metabolic fecal nitrogen. Neither of these techniques is applicable to the measurement of "true digestibility" during a prolonged period of growth.

Titus ('27) determined the "true digestibility" of alfalfa protein in steers by means of a different technique. The protein content of the ration was varied and the total food intake was maintained constant. The total nitrogen intake was plotted as a function of the total fecal nitrogen excretion. The resulting straight line was extrapolated to the point of zero nitrogen intake, the Y intercept then being used as the metabolic fecal nitrogen excretion. The suggestion was made that in steers, the fecal nitrogen excretion on a nitrogen-free diet could not safely be taken as a measure of metabolic fecal nitrogen when the animals were ingesting protein.

Schneider ('35) has shown that the metabolic fecal nitrogen fraction consists of two components: a constant fraction that is proportional to the body surface area, and a variable fraction that is proportional to the quantity of dry food consumed. The variation due to differences in the quantity of food consumed is corrected for by expressing metabolic fecal nitrogen as a function of food consumption, i.e., mg. of metabolic fecal nitrogen per 100 gm. of food or dry matter consumed.

The object of this study was to investigate an extrapolation procedure for estimating metabolic fecal nitrogen, using mice as test animals.

#### EXPERIMENTAL METHOD

Male albino mice (Sharp and Dohme, Swiss-Webster strain) were used in these studies. In the one study involving restricted feeding mature mice weighing from 20 to 30 gm. were used. In the experiments involving growth, mice weighing 7 to 9 gm. at 15 to 16 days of age were selected and subjected to a preliminary adjustment period of 2 days (Bosshardt et al., '45). In all experiments, the mice were housed in individual wire-bottom cages and supplied water ad libitum. The feces for each group were pooled for the entire test period in 10% sulfuric acid. Nitrogen determinations were made by a modified Kjeldahl procedure in which the total sample was di-

gested partially by refluxing with 20% sulfuric acid after which aliquot portions were used for the final digestion and distillation.

The protein sources studied were: acetone extracted, heat coagulated whole egg, casein,<sup>1</sup> and wheat gluten. They were incorporated at the different levels into a basal ration that consisted of 25% hydrogenated cottonseed oil (Primex), 2% corn oil,<sup>2</sup> 20% dextrose,<sup>3</sup> 4% salt mixture (Hubbell, Mendel, and Wakeman, '37), 2% cellu flour, 1% Wilson's 1:20 liver concentrate powder and sufficient white dextrin to make 100%. All diets were supplemented to contain per 100 gm.: 4 mg. of alpha-tocopherol, 900 U.S.P. units of vitamin A, 180 U.S.P. units of vitamin D, 1 mg. of 2-methyl-, 4-naphthoquinone diacetate, 0.8 mg. of thiamine hydrochloride, 1.6 mg. of riboflavin, 0.8 mg. of pyridoxine hydrochloride, 4.0 mg. of niacin, 4.4 mg. of calcium pantothenate, 4.0 mg. of para-aminobenzoic acid, 21.6 mg. of inositol, and 200 mg. of choline chloride. In the study with restricted food intake, the liver concentrate powder was omitted.

### *Digestibility with restricted food intake*

The data in this experiment were obtained in a study undertaken to determine the effect of protein feeding on body protein loss when mice were restricted to approximately 30% of their normal caloric intake. The diets contained extracted whole egg at eight levels ranging from 0% to 40% (table 1). Each diet was fed to a group of eight mice in an amount of 1.0 gm. per mouse per day for 14 days.

The relationship between nitrogen intake per 100 gm. of food consumed and fecal nitrogen per 100 gm. of food consumed is shown in figure 1. As plotted, the data, with the exception of that obtained with the protein-free diet

<sup>1</sup> Borden's "Labco" casein.

<sup>2</sup> Mazola.

<sup>3</sup> Cerelease.

approximate a straight line. The straight line of best fit was calculated from the equation,  $Y = a + b$  where

$$b = \frac{[\Sigma (xy)] n - \Sigma x \cdot \Sigma y}{[\Sigma (x^2)] n - [\Sigma (x)]^2} \quad \text{and} \quad a = \frac{\Sigma y - b \Sigma x}{n} \quad \text{or} \quad a = \bar{y} - b\bar{x}$$

The standard deviation of  $a$  was calculated according to the equation

$$\sigma a = \sqrt{\frac{\Sigma x^2 (\Sigma y^2 - a \cdot \Sigma y - b \cdot \Sigma xy)}{n [n - 2 (\Sigma x^2 - n\bar{x}^2)]}}$$

The value obtained with the protein-free diet was omitted from the calculation because of its marked deviation from the straight line, a finding that tends to confirm the observation of Titus ('27) that the fecal metabolic nitrogen excretion on a protein-free diet may not be the same as when protein is being consumed.

The extrapolation of this line to the point of zero nitrogen intake gave a value of 322.9 mg. of metabolic fecal nitrogen per 100 gm. of food consumed. The value obtained with the protein-free diet was 300 mg. per 100 gm. of food consumed. The standard deviation of the extrapolated value was  $\pm 3.7$ . This is an indication that with mice the metabolic fecal nitrogen is affected by the feeding of protein and erroneous results may be obtained by using a value yielded when protein-free diets are fed. The calculated "true digestibility" of the whole egg protein, using the correction of 322.9 mg. of fecal nitrogen per 100 gm. of food consumed for this experiment, was  $95.4 \pm 0.2\%$ .<sup>4</sup>

### *Digestibility with ad libitum food intake*

The relationships between nitrogen intake and fecal nitrogen per 100 gm. of food consumed for mice ingesting the three proteins ad libitum are shown in figure 2. The metabolic fecal nitrogen values obtained by extrapolation were: whole egg, 221 mg. per 100 gm. of food consumed, and for wheat gluten, 217 mg. per 100 gm. of food consumed. The data for the

<sup>4</sup> Standard error =  $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$

casein-fed animals included one anomalous value—that obtained with the lowest level of nitrogen intake. The fecal nitrogen per 100 gm. of food consumed actually found by analysis was 189 mg., whereas the value calculated from the straight line was 244 mg. The standard deviation of the Y intercept was found to be  $\pm 7.1$  which is an indication that the observed difference was highly significant. This value therefore was omitted in the calculations of the Y intercept and average digestibility.

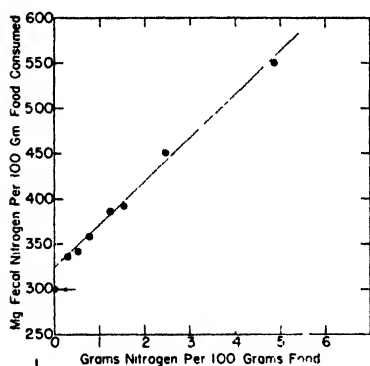


Fig. 1 The relationship between nitrogen intake and fecal nitrogen excretion with varying levels of whole egg protein in the diet. The food intake was restricted to approximately 30% of the normal caloric requirement.

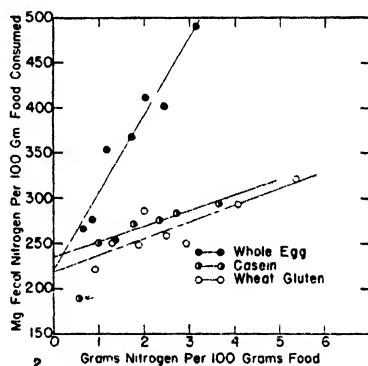


Fig. 2 The relationship between nitrogen intake and fecal nitrogen under conditions of ad libitum feeding with varying protein levels in the diet.

The casein-fed group showing the anomalous result did not grow throughout the feeding period. If, as these data suggest, the metabolic fecal nitrogen when the animal is just in nitrogen equilibrium is not the same as when the animal is in positive nitrogen balance, as is the case during growth, values obtained under one set of conditions are not necessarily adaptable to an entirely different set of conditions.

The "true digestibilities" at the different levels of intake of the proteins studied are shown in table 1. The two sets of data obtained with whole egg feeding confirm the results of Barnes et al. ('45), who reported an increase in digesti-



TABLE 1

*Protein digestibilities with varying levels of intake*

WHOLE EGG-RESTRICTED <sup>1</sup>		WHOLE EGG AD LIBITUM <sup>2</sup>		OASEIN AD LIBITUM <sup>3</sup>		WHEAT GLUTEN AD LIBITUM <sup>4</sup>	
N intake per 100 gm. diet <sup>5</sup>	"True digestibility"	N intake per 100 gm. diet	"True digestibility"	N intake per 100 gm. diet	"True digestibility"	N intake per 100 gm. diet	"True digestibility"
gm	%	gm	%	gm.	%	gm.	%
0.287	95.5	0.660	92.9	1.000	98.4	0.920	99.8
0.532	96.4	0.870	93.4	1.360	98.7	1.300	97.6
0.770	95.5	1.190	88.7	1.780	98.0	1.870	98.9
1.220	94.9	1.730	91.4	2.350	98.3	2.000	96.6
1.540	95.5	2.020	90.4	2.710	98.2	2.480	98.4
2.480	94.9	2.440	92.5	3.630	98.4	2.930	98.9
4.890	95.4	3.140	91.3	..	..	4.080	98.2
..	..	..	..	..	..	5.370	98.1
95.4 ± 0.2 <sup>6</sup>		91.5 ± 0.6		98.3 ± 0.1		98.3 ± 0.3	

<sup>1</sup> Metabolic fecal nitrogen = 323 mg./100 gm. food consumed.<sup>2</sup> Metabolic fecal nitrogen = 221 mg./100 gm. food consumed.<sup>3</sup> Metabolic fecal nitrogen = 235 mg./100 gm. food consumed.<sup>4</sup> Metabolic fecal nitrogen = 217 mg./100 gm. food consumed.<sup>5</sup> Equivalent to per cent nitrogen in the diet.

$$^6 \text{Standard error} = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$$

bility when the protein intake was restricted by paired feeding as compared with ad libitum feeding.

## DISCUSSION

In studies of protein nutrition an estimation of the "true digestibility" often is desired. It is recognized that nitrogen in the feces originates from two sources: unabsorbed food nitrogen and nitrogen resulting from body metabolism and excreted by way of the feces. The fecal nitrogen excretion on a protein-free diet frequently has been used as a measure of the metabolic fecal nitrogen. This is based on the assumption that the metabolic fecal nitrogen is dependent upon body surface area and the amount of dry food consumed and is not influenced by the presence of protein in the food. The extrapolation procedure is an attempt to determine the metabolic nitrogen of the feces under conditions of protein feeding.

The data in this report indicate, as do the results of Titus ('27), that the fecal nitrogen excretion on a protein-free diet is not a safe measure of metabolic fecal nitrogen when protein is included in the diet.

When the nitrogen intake per unit of food consumed at several different levels of intake is plotted as a function of the fecal nitrogen per unit of food consumed, the plot of the data fits very well a straight line. If a protein is completely digested and absorbed this line should be parallel to the X axis. As the slope of this line increases with different proteins, the "true digestibility" decreases.

The data indicate that whole egg protein is not completely digestible, a finding that is contrary to the results of Mitchell and Carman ('24). This may be due to a species difference between the mouse and the rat; however, the results of Barnes, Maack, Knights and Burr ('45), which were obtained with rats, indicate that wheat gluten is digested more completely than is whole egg protein.

Another interesting point is the increase in "true digestibility" of egg protein when the food intake is restricted. This appears to be similar to other cases in which the degree and rate of absorption is influenced by the state of deficiency or it may be due in part to the peculiar absorption pattern that is found when animals consume their daily food allotment in a comparatively short time.

It follows from the work of Schneider that the metabolic fecal nitrogen when expressed as a function of food consumed should increase when food consumption is restricted. This has been explained as evidence of a second factor that involves intestinal secretion and is related to body size. The observation that a straight line relationship exists between the nitrogen content of the ingested food and the fecal nitrogen per unit weight of food consumed indicates that there is no serious deviation in the relative contribution of these two factors toward the total metabolic fecal nitrogen under conditions of protein feeding. However, under conditions of severe protein restriction or the ingestion of a protein-free diet it is possi-

ble that the intestinal secretion of nitrogen is markedly decreased. This would result in an apparent lowering of the metabolic fecal nitrogen.

#### SUMMARY

“True digestibilities” of the proteins of whole egg, wheat gluten, and casein and the fecal nitrogen of metabolic origin have been determined under conditions of protein feeding with growing mice. The metabolic fecal nitrogen was determined by plotting the nitrogen intake per unit of food consumed at different dietary protein levels as a function of the fecal nitrogen excretion per unit of food consumed. The calculated straight line was extrapolated to the point of zero nitrogen intake. The Y intercept was then used as the value for the metabolic fecal nitrogen per unit of food consumed.

Under conditions of ad libitum feeding the average “true digestibilities” determined by this method were: casein  $98.3 \pm 0.1\%$ , wheat gluten  $98.3 \pm 0.3\%$ , and extracted whole egg  $91.5 \pm 0.6\%$ . When the food intake of mature mice was restricted to approximately 30% of the normal caloric requirement the average “true digestibility” of extracted whole egg was found to be  $95.4 \pm 0.2\%$ .

The data indicate that metabolic fecal nitrogen values determined with protein-free or low protein diets are not safe indices of the metabolic fecal nitrogen under conditions of protein feeding.

#### ACKNOWLEDGMENT

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# THE USE OF MICE FOR THE MEASUREMENT OF THE GROWTH PROMOTING QUALITY OF PROTEINS

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## FIVE FIGURES

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Probably the most extensively used method for the determination of the nutritive quality of proteins for growth is the protein efficiency method of Osborne, Mendel and Ferry ('19). The nutritive quality of a protein is expressed as the ratio of body weight gain to protein consumed determined with growing rats that are fed the test protein under specified conditions. As commonly employed, this procedure has been subjected to numerous modifications by different workers. This lack of a standardized procedure makes it difficult to compare and evaluate the results from different laboratories.

The relative growth rate of the albino mouse is approximately twice that of the rat. If the protein efficiency method could be adapted to the mouse as the test animal it should be possible to decrease the time necessary for a growth measurement which, coupled with the smaller food requirements, would result in a saving of time, labor, and dietary ingredients.

The purpose of this study was to establish a rational procedure for the use of mice for protein efficiency determinations and to investigate the effect of such factors as pre-test standardization of test animals, the duration of the feeding period, and the level of the test protein in the diet.

## EXPERIMENTAL METHOD

Male, weanling, albino mice (Sharp and Dohme, Swiss-Webster strain) were used in all studies. The mice were removed from the stock colony at the age of 15 to 16 days. Although the weight range at this age was 5.5 gm. to 9.5 gm., only those mice weighing from 7.0 gm. to 9.0 gm. were used. They were placed in individual wire bottom cages, and given water and the test diets *ad libitum*. All of the test periods were of 20 days' duration. To facilitate the recovery of feces and spilled food, with a minimum of urine contamination, blotting paper was placed below the cages. Daily records were kept of the animal weights and food consumption. All weighings were made to the closest 0.1 gm. The feces for each test group were pooled for the entire test period in 10% sulfuric acid. At the conclusion of each test period, the mice were killed with gas, and the carcasses saved for total body nitrogen determinations.

Preliminary experiments have shown that a fasting period of from 3 to 4 hours is sufficient for an almost complete emptying of the intestinal tracts of mice. Therefore, in order to eliminate the nitrogen in the intestinal contents in the determination of body nitrogen, the animals were killed 3 hours after the removal of the food from the cages at the last weighing. It was felt that this method would involve a smaller error than would a washing procedure.

Total nitrogen determinations of the diets, feces, and carcasses were made by the Kjeldahl method with modifications depending upon differences in sample sizes. For all determinations, the entire samples were partially digested and aliquot portions were used for the final digestion and distillation procedures.

The three protein sources used in these studies were acetone extracted, heat coagulated whole egg, casein,<sup>1</sup> and wheat gluten. They were incorporated into a basal ration which consisted of 25% hydrogenated cottonseed oil,<sup>2</sup> 2% corn oil,<sup>3</sup>

<sup>1</sup> Borden's "Labco" casein.

<sup>2</sup> Primex.

<sup>3</sup> Mazola.

20% glucose,<sup>4</sup> 4% salt mixture (Hubbell, Mendel and Wakeman, '37), 2% cellu flour, 1% Wilson's 1:20 liver concentrate powder, and sufficient white dextrin to make 100%. All diets were supplemented to contain per 100 gm.: 4 mg. of alpha tocopherol, 900 U.S.P. units of vitamin A, 180 U.S.P. units of vitamin D, 1 mg. of 2-methyl-1, 4-naphthoquinone diacetate, 0.8 mg. of thiamine hydrochloride, 1.6 mg. of riboflavin, 0.8 mg. of pyridoxine hydrochloride, 4.0 mg. of niacin, 4.4 mg. of calcium pantothenate, 4.0 mg. of para-aminobenzoic acid, 200 mg. of choline chloride, and 21.6 mg. of inositol. This diet has been shown by Bosshardt et al. ('45b) to support good growth of mice.

*The effect of the pre-test treatment of the animals*

It has been observed that a change in the environment of the growing mouse causes a definite break in the growth curve. When weanling mice are transferred from the stock colony to the individual test cages they always show a loss of body weight the first day. The time required to regain this loss has been found to vary with the diet. When Purina Fox Chow or purified diets containing 10% or 20% casein were fed, the initial weight was regained after 4 days. When a purified diet containing 15% extracted whole egg was fed, the initial weight was regained in 2 to 3 days.

Four groups of seven male weanling mice were fed a test diet containing 10% casein for 20 days following each of the four pre-test treatments: (A) no holding period, (B) a 2-day pre-test period with a purified diet containing 15% extracted whole egg, (C) a 4-day pre-test period with Purina Fox Chow, and (D) a 4-day pre-test period with a purified diet containing 20% casein. The average growth curves and the protein efficiency ratios calculated at 10 days and at 20 days are shown in figure 1. Although the growth curves have the same slope, there is a marked difference between the protein efficiency ratios obtained after a preliminary 2-day feeding period

<sup>4</sup> Cerelese.



with a 15% extracted whole egg diet and those obtained after the other pre-test treatments. This difference is more pronounced for the values calculated at 10 days than for the values calculated at 20 days. These data indicate that growth rates are not reliable indices of protein utilization. The differences observed in the protein efficiency ratios indicate that

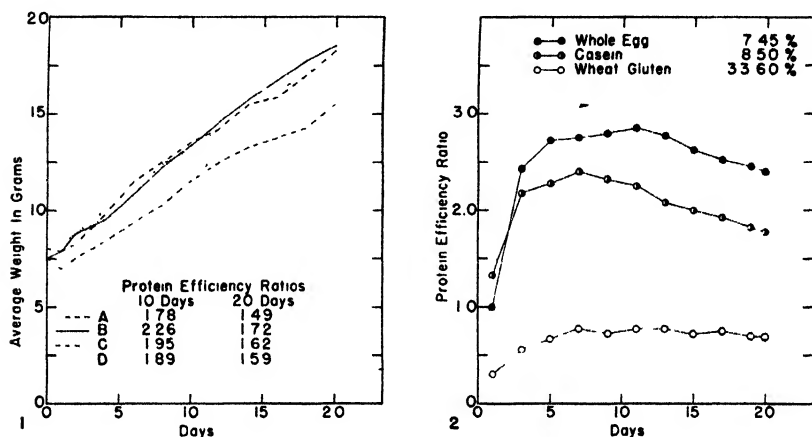


Fig. 1 The effect of pre-test treatment on the growth and protein efficiency ratios of mice receiving a purified diet containing 10% casein.

Fig. 2 The relation between time and protein efficiency ratios.

following a 4-day period of body weight loss and regeneration a greater protein intake is necessary to attain a definite growth rate than is required following the shorter 2-day period of body weight loss and regeneration.

As a result of these findings, in all subsequent studies a 2-day pre-test standardizing period with a purified diet containing 15% extracted whole egg was employed. This procedure was chosen because it was felt that, although all animals had gone through a short period of depletion and regeneration, any adverse effect would be minimized with the shorter period. As a further refinement, only those mice that had come to within 0.5 gm. or less of attaining their initial weight after the 2-day pre-test period were used.

*The effect of the duration of the test period*

The three protein sources, extracted whole egg, casein, and wheat gluten were incorporated into the basal ration to supply protein levels of 7.45%, 8.50%, and 33.5% ( $N \times 6.25$ ), respectively.<sup>5</sup> Twenty-day growth studies were made with groups of seven mice each that had been selected as previously described. The protein efficiency ratios were calculated daily throughout the experiment. The results are shown in figure 2. The optimal time for most constant results appears to be between 7 and 12 days since during this period there was a tendency for the slope of the curves to decrease. As the periods were continued beyond 12 days there was a gradual but definite decrease in the protein efficiency ratios of the better proteins. This was due to a slight decrease of the growth rate that occurred after 10 to 12 days with these diets. Since the food consumption, and thus the protein intake, did not decrease but gradually increased, the protein efficiency ratio decreased. Although all experiments in this report were of 20 days' duration, routine protein efficiency ratio determinations in our laboratories now are calculated at the end of 10 days.

*The effect of the level of the test protein in the diet*

When expressing the nutritive quality of a protein as its protein efficiency ratio, Osborne, Mendel, and Ferry specified the level of protein in the diet at which the maximal ratio was obtained. They suggested that this dietary level be established for each protein tested. In the most generally employed modifications of the method a single level of test protein, usually approximating 10%, is employed. As has been shown by Osborne, Mendel, and Ferry and by Barnes, Maack, Knights, and Burr ('45) the level of the test protein in the diet at which the maximal protein efficiency ratio is obtained varies with different proteins. Their data indicate that pro-

<sup>5</sup> These levels were selected from data presented in the following section as approximating those showing optimal protein efficiency ratios.

teins of poorer quality must be fed at relatively high levels to obtain the maximal protein efficiency ratios.

The three protein sources were incorporated into the basal ration, each at eight different levels ranging from 3% to 40%. Each diet was fed for 20 days to seven mice that had been selected as previously described. The protein efficiency ratios calculated at 10 days and at 20 days are shown in figures 3 and 4. The dietary level at which maximum utilization for growth was obtained was very definite for the better quality proteins,

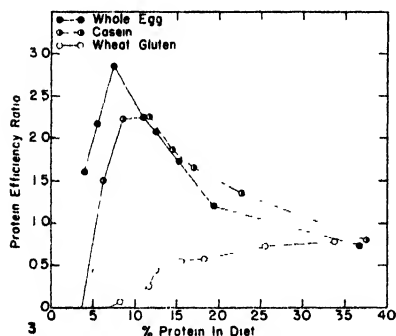


Fig. 3 The effect of the protein level in the diet on protein efficiency ratios at the end of a 10-day feeding experiment.

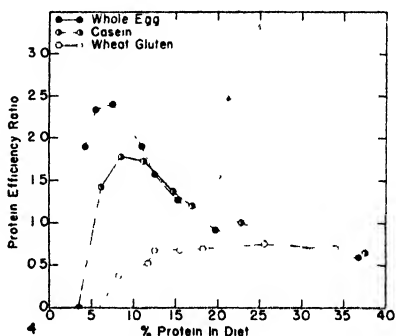


Fig. 4 The effect of the protein level in the diet on protein efficiency ratios at the end of a 20-day feeding experiment.

extracted whole egg and casein. This point was not so definite for the poorer quality protein, wheat gluten. These data tend to confirm the results mentioned earlier which were obtained with rats and which indicate that the poorer the protein the higher must be its level in the diet for maximal utilization for growth.

Although all growth studies included in this report are of 20 days' duration, the marked similarity existing between the curves calculated at 10 days and at 20 days, figures 3 and 4, indicates that valid results may be obtained by using a 10-day test period.

In expressing protein utilization for growth as the protein efficiency ratio, body weight gain is used as an index of body

protein gain. This gives rise to a criticism of the method. The protein content of the growing animal is not a constant factor. Weanling mice after the 2-day pre-test period have been found to contain  $2.54 \pm 0.02\%$  N.<sup>6</sup> The body nitrogen levels and the average body weight and body nitrogen gains after the 20-day feeding periods are shown in table 1. It is evident that weight gain is not necessarily a true index of body nitrogen gain. The most striking example is that shown

TABLE 1

*The body weight and body nitrogen gains and body nitrogen levels of mice receiving various diets for 20 days.*

EXTRACTED WHOLE EGG				CASEIN				WHEAT GLUTEN			
Nitro- gen in diet	Aver- age body weight gain	Aver- age body nitro- gen gain	Nitro- gen in car- casses	Nitro- gen in diet	Aver- age body weight gain	Aver- age body nitro- gen gain	Nitro- gen in car- casses	Nitro- gen in diet	Aver- age body weight gain	Aver- age body nitro- gen gain	Nitro- gen in car- casses
%	gm	mg.	%	%	gm	mg	%	%	gm	mg	%
0.66	3.94	129	2.79	0.57	0.0	8	2.66	0.92	-0.13	12	2.75
0.87	7.01	217	2.80	1.00	4.1	137	2.72	1.30	1.11	43	2.72
1.19	11.22	339	2.82	1.36	9.0	286	2.88	1.87	2.20	74	2.74
1.73	12.90	396	2.86	1.78	12.6	357	2.74	2.00	3.49	110	2.74
2.02	12.68	397	2.91	2.35	12.3	379	2.86	2.48	4.41	144	2.82
2.44	12.28	412	3.05	2.71	12.9	432	3.05	2.93	5.56	180	2.85
3.14	10.89	452	3.42	3.63	13.6	435	2.96	4.08	9.41	288	2.83
5.86	12.93	402	2.90	6.00	13.5	428	2.94	5.37	10.41	336	2.94

by the three highest levels of whole egg protein. At dietary nitrogen levels of 2.44%, 3.14%, and 5.86% the average weight gains were 12.3 gm., 10.9 gm., and 12.9 gm., respectively. The average body nitrogen gains, however, were 412 mg., 452 mg., and 402 mg., respectively. The body nitrogen gains were determined as the difference between the level at the start of the experiment (weight  $\times$  2.54%) and the determined level at the conclusion of the experiment. Under the conditions of protein feeding at the level giving maximal utilization for

$$^6 \text{Standard error} = \sqrt{\frac{\sum d^2}{n(n-1)}}$$

growth the body nitrogen levels were essentially the same for the three proteins: extracted whole egg 2.82%, casein 2.88%, and wheat gluten 2.83%. Therefore, at dietary protein levels at which maximal protein efficiency ratios are obtained body weight gains may be used as indices of comparative body protein gains. However, as the body protein level changes during a growth study the use of body weight gains even at the dietary level showing maximum utilization for growth cannot be used as absolute indices of the utilization of ingested or absorbed protein for body protein gain.

A more valid expression of protein utilization for growth would be the utilization of ingested or, preferably, absorbed protein for body protein gain. Protein intake under conditions of ad libitum feeding may be varied by varying the protein level of the diet. The determination of body nitrogen gains at different levels of protein intake makes it possible to compare different proteins at equal intake levels using ad libitum feeding. It is also possible to determine the level of intake at which maximal utilization for growth is obtained. If "true digestibilities" are used, the calculations can be based on absorbed protein.

The "true digestibilities" of the three proteins were determined as described by Bosshardt and Barnes ('45a). The amount of fecal nitrogen per 100 gm. of food consumed was plotted against the nitrogen intake per 100 gm. of food at the different levels of protein intake. The calculated straight line was extrapolated to the point of zero nitrogen intake to obtain the metabolic fecal nitrogen value under conditions of protein feeding. The average "true digestibilities" obtained were: extracted whole egg  $92.5 \pm 0.6\%$ , casein  $98.3 \pm 0.1\%$ , and wheat gluten  $98.3 \pm 0.03\%$ .

The relationship between absorbed protein and the percentage of absorbed protein utilized for body protein gain for the three proteins during the 20-day test period is shown in figure 5. This method of calculation considers differences in food consumption, and thus in protein intake, that are omitted in figures 3 and 4. These data indicate that there is a

shift of the point of maximal utilization for growth to higher levels of protein absorption accompanying a decrease in the nutritive quality of the protein. It is, however, possible to compare the three proteins at a level of absorption approximating utilization for each. An approximate maximal utilization was observed when 5 gm. of each protein were absorbed per mouse for the 20-day period. This level of protein absorption would correspond to diets containing approximately 7.5% of extracted whole egg, 8.5% of casein and 12.5% of

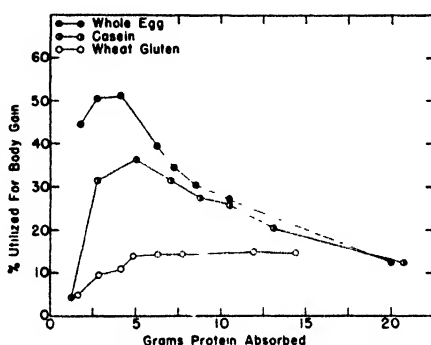


Fig. 5 The utilization of absorbed protein for body protein gain at different levels of protein intake.

wheat gluten. The true dietary levels for maximal utilization would be approximately 6.5% of extracted whole egg, 8.0% of casein, and 25% of wheat gluten corresponding to absorptions of 3.5 gm., 4.5 gm., and 12.0 gm., respectively.

#### DISCUSSION

The data presented in this report indicate that the albino mouse is a suitable test animal for the determination of the nutritive quality of proteins for growth. The advantages of using the mouse instead of the rat are several. A reliable growth result may be obtained in 10 days. Thus a marked saving of time is realized since the conventional test period for the rat method is 28 to 42 days. Approximately one-seventh the amount of test protein and other dietary ingredients are needed because of the saving in time and the smaller food

consumption of the mouse as compared with the rat. The saving in time and dietary ingredients realized by the use of the mouse will facilitate fractionation and chemical studies that are dependent on animal assays. The mouse likewise lends itself to studies involving expensive materials or those difficult to prepare such as isotopically labeled compounds. The reduced time necessary for a study usually makes it unnecessary to prepare more than one lot of diet, which is an aid in assuring constancy of the diet. For studies involving the whole carcass, the size of the mouse lends itself to simplified procedures for total carcass analysis.

The absolute efficiency ratios are not necessarily the same for both rats and mice, but the results of a large number of assays indicate that proteins and protein hydrolysates fall in the same order of classification by the two methods.

Weight changes and food consumption for the mouse are considerably less than for the rat, so that measurements must be made more accurately. The data indicate that there are certain factors that can affect the result in protein efficiency measurements when the mouse is used as the test animal. Similar effects may be noted when other animal species are employed. Factors such as pre-test standardization of the animals, the duration of the test period, and the level of the test protein in the diet can be and should be standardized if it is to be possible to compare and evaluate results from different laboratories. Differences in the reported numerical nutritive indices of proteins may be due, at least in part, to differences in experimental technique. Other factors possibly influencing protein utilization measurements that should be investigated are caloric intake, ratios of fat and carbohydrate in the test diets, nature of the mineral and vitamin supplementations, amount of undigestible material in the ration, and the strain of the animal species employed.

#### SUMMARY

The method of Osborne, Mendel and Ferry for the measurement of protein efficiency ratios (grams gain in body weight

per gram of protein consumed) has been adapted for use with mice.

The importance of establishing standardized conditions with regard to the pre-test treatment of the test animals, the duration of the test period, and the level of test protein in the diet has been demonstrated.

The advantages of the use of the mouse as compared with the rat are: smaller animal size, lower food consumption, and shorter test periods.

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# ENERGY AND GASEOUS METABOLISM OF THE CHICKEN FROM HATCH TO MATURITY AS AFFECTED BY TEMPERATURE

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FOUR FIGURES

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## INTRODUCTION

Metabolic activity is measured by energy output. Energy is required for work, for digestion and assimilation of feed-stuffs and also to maintain body temperature. Since the energy expended to maintain body temperature is dependent on temperature of environment, this factor could have a profound influence on metabolic activity and thus on the feed consumption and meat and egg production of the chicken.

In a previous investigation, Barott and Pringle ('41) studied the effect of different environmental temperatures on the metabolism of the hen. In the investigation herein reported, the program of work was enlarged to include the life cycle of chickens from hatch to maturity, and a range of temperature from 20°F. to 103°F.

## APPARATUS AND PROCEDURE

The data at temperature of 40°F. and above were obtained by use of one of the respiration calorimeters in the calorimetry laboratory of the Bureau of Animal Industry, Agricultural Research Center, Beltsville, Maryland. The details of construction of the calorimeter and technique of operation were

described by Barott ('37). It was necessary to alter the calorimeter for work at temperatures below 40°F. to a low temperature respiration chamber because the temperature control system could no longer function below this temperature. This alteration was accomplished by removing the heat absorber coil and inserting a cooling unit connected to a small refrigerating machine. The heating and cooling coils for the boundary control were also removed and 2 inches of cork insulation were added around the chamber. Therefore it was impossible to measure heat loss at temperatures below 40°F. It was also impossible to determine the water elimination because of the deposition of the moisture in the form of frost on the refrigeration unit. Accurate measurements of oxygen consumption and carbon dioxide elimination were obtained, however.

The procedure was similar to that with hens (Barott and Pringle, '41), except that the baby chicks and chicks 2 weeks of age were placed in a cage made of fine-meshed copper screen, divided into compartments. Each compartment was sufficiently large to accommodate one chick. While freedom of movement was not so severely restricted as in the case of the older birds no huddling was possible. Paper towels were put under the cages in the calorimeter to catch the down and any other material that came from the chicks and passed through the cages. This was done so that an accurate weight of the chicks could be determined. The weight of the feathers, droppings, etc., were subtracted from the beginning weight as noted under "Results."

The chickens 5 weeks, 8 weeks, and 12 weeks of age were put in individual galvanized wire cages. These cages were placed in the calorimeter over a pan of oil to catch the droppings. The chickens older than 12 weeks were placed in the calorimeter in individual cages similar in construction to those used for hens (Barott and Pringle, '41) except that they were smaller. These cages were also set over oil.

The chickens used were Rhode Island Red females. They were kept in battery brooders and fed the all-mash starting and growing diet no 2 (Titus, '41). The hens were fed a nor-

mal laying diet, and were housed in a partially open front laying house of the type used at this station (Mohler, '39).

The chickens were brought to the laboratory at 5 A.M., so that little, if any, feed was consumed since the previous night. They were weighed at the beginning and end of each experiment. During the time they were in the calorimeter they were in complete darkness and had no feed or water.

During the experimental period the air temperature within the calorimeter was kept practically constant at a pre-determined value, the relative humidity was kept between 50 and 60%, the oxygen content at 21% and the carbon dioxide below 1%.

A total of more than 500 1-day experiments were performed at approximately 5°F. intervals within a range of temperature from 20°F. to 103°F. The range limit for each age studied was determined by the survival of the chickens.

The oxygen consumption was measured for each 2-hour period and the carbon dioxide, water, and heat elimination for each 4-hour period.

The investigation was started with two groups of baby chicks, 2 to 6 days old, 100 in each group, hatched 1 week apart. No chickens from either group were used for more than 1 day's experiment at any one age. After the day's experiment they were put back into the brooder or on the range to grow until they reached the next age to be studied.

The ages investigated were decided upon after a study of the growth curve (fig. 2) which indicated that there might be greater changes occurring in the metabolic rate for the chickens up to 12 weeks of age than in that of the older ones. Therefore studies were made using baby chicks, and chickens 2 weeks, 5 weeks, 8 weeks, and 12 weeks old. As chickens begin laying at 4 to 6 months of age, the next age studied was 18 weeks, then 23 weeks, and finally 1 year. This range of age covers the growth period fairly well.

The data on oxygen consumption, and heat, water and carbon dioxide elimination obtained during the experimental period were computed, compiled and prepared for analysis for each

individual experiment. All volumetric values for oxygen consumption and carbon dioxide elimination were reduced to a common basis: 0°C. and 760 mm. atmospheric pressure.

In the analysis of the data the question arose as to what basis of comparison to adopt, i.e., whether to express the results as a function of body surface of the animal or of some power of the body weight. It is hard to determine with accuracy the body surface of a live animal, especially one covered with feathers. However, it is very easy to obtain an accurate weight. As metabolic activity depends upon the amount of active protoplasm in the living cells of the body, it is difficult for the authors to see wherein the body surface factor is any more accurate, if as accurate, as the body weight and their view is upheld by other investigators. Kleiber ('32) in an exhaustive treatise on the subject, states in his conclusions: "A power function of the body weight gives a better defined unit for measurement than the unit of body surface." Therefore, all results in this investigation are expressed as a function of the body weight and since there is no agreement among investigators as to what the power coefficient of the weight should be, we have expressed all results as a function of the weight to the first power. The initial weight of the bird, minus the weight of the excrement, was used in computing the initial value for energy and gaseous metabolism. The final weight was used in computing the final values. For computing the intermediate values, the difference between these two weights was pro-rated linearly with time.

#### RESULTS

The results of our previous work on hens (Barott and Pringle, '41) were shown by curves. The hens used in those experiments varied in weight from 1800 to 2800 gm.; however, in plotting the curves no account was taken of the variation in metabolism per gram due to this difference in the size of the hen. When the data on hens were analyzed with due regard for variation in weight, the double flexure previously shown in the curve for metabolic activity disappeared and the curve

assumed the form shown in this paper. The results previously published (Barott and Pringle, '41), when adjusted for variations in weight agree very well with those shown herein.

#### ENERGY AND GASEOUS METABOLISM

Values for oxygen consumption and for carbon dioxide and heat elimination were computed from observed data for each experiment. All the results for experiments at one temperature, with chickens of the same weight, were plotted on one chart and a curve drawn through the plotted points. This curve represented the metabolic activity of a chicken of given weight at one definite temperature. In figure 1, the oxygen consumption at  $70^{\circ}\text{F.} \pm 2.0^{\circ}\text{F.}$  of chickens of various ages is shown. The curves (fig. 1) all have the same general form for metabolic activity for the 24-hour period and are of the same type as those previously published. They all show the typical diurnal rhythm (first reported from this laboratory, Barott et al., '38) with a high value for metabolic rate at 8 A.M., gradually declining to a low at 8 P.M. and thereafter rising to another high at 8 A.M. the next day.

Normally, the value at 8 A.M. the second day would be of the same magnitude as that of the first day. However, because the chickens had no feed or water for the duration of the experiment, the second day's value is 5 to 10% lower except for baby chicks and for them the value the second day is the same as that for the first day because of the unabsorbed yolk upon which the chick feeds.

The magnitude of the metabolic activity when expressed per gram weight of chicken per hour is greatest for baby chicks and becomes progressively less and less as the chick gets older. It is least for the oldest chickens. However, the total metabolism (the value in units per hour per gram weight of chicken, times the weight of the chicken in grams), is least for the baby chicks and becomes greater very rapidly as the chicken's weight increases from 40 gm. to 300 gm. The rate of increase then becomes less and less as the age and weight

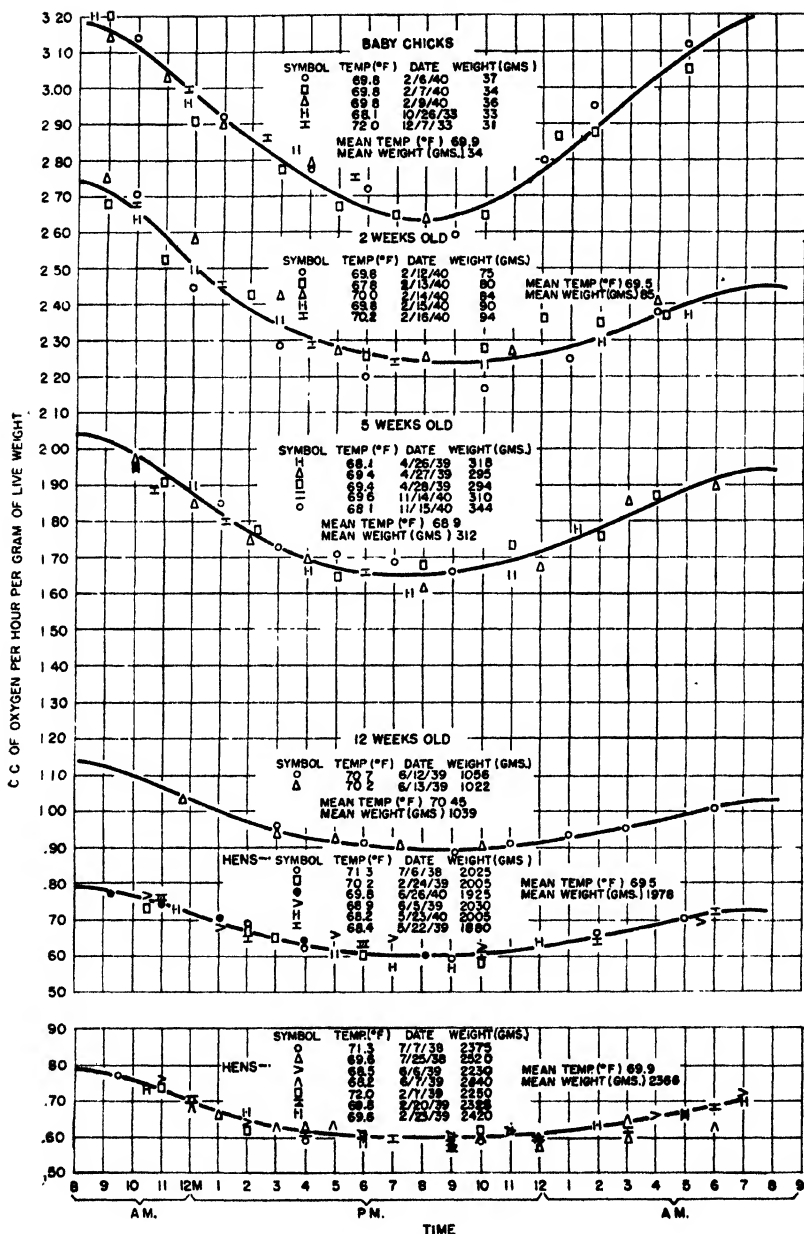


Fig. 1 Oxygen consumption of chickens of various ages at an environmental temperature of 70°F.

of the chicken increase, until it is practically zero at weights over 2800 gm.

By integration of curves similar to those in figure 1, the mean metabolic rate between 8 A.M. and 8 P.M. was determined for each experiment. The values thus obtained were plotted (fig. 2) and curves drawn through the plotted points in such a manner that they represent the mean value of the plotted points as closely as possible. These curves delineate the mean metabolic rate for each 10-degree interval from 30° to 90°F. Curves for 70°, 80°, and 90°F. delineate the metabolic rate for the life cycle of the chick from hatch to maturity.

Curves below 70°F. extend to the weight and age at which the chicken could survive the temperature studied. For example, baby chicks and chicks 2 weeks old did not survive 24 hours at temperatures below 70°F., those 5 weeks old did not survive below 50°F., etc. The lowest survival temperature for chickens older than 8 weeks has not yet been determined.

It will be noted that the trend of all curves is the same and that the metabolic rate decreases as the age and weight of the chicken increase. This decrease is very rapid during the first few weeks of growth, but becomes less and less until, between the weights of 2400 and 2800 gm., the rate is nearly constant.

Curves for carbon dioxide and heat elimination have the same form as those for oxygen consumption and differ only in absolute value. They have been omitted for the sake of brevity, as have those for oxygen consumption, and carbon dioxide and heat elimination for temperatures of 25°, 35°, 45°, 55°, 65°, 75°, and 85°F. However, all these curves were plotted and analyzed so that the curves shown in figure 3 could be constructed from the values.

Each curve in figure 3 shows the variation in metabolic rate (mean oxygen consumption between 8 A.M. and 8 P.M.) with temperature for a given weight of chicken. These curves (fig. 3) cover the life cycle of the chicken from the time of hatch to maturity. The values plotted on these curves were taken from figure 2 and similar curves. The carbon dioxide elimina-



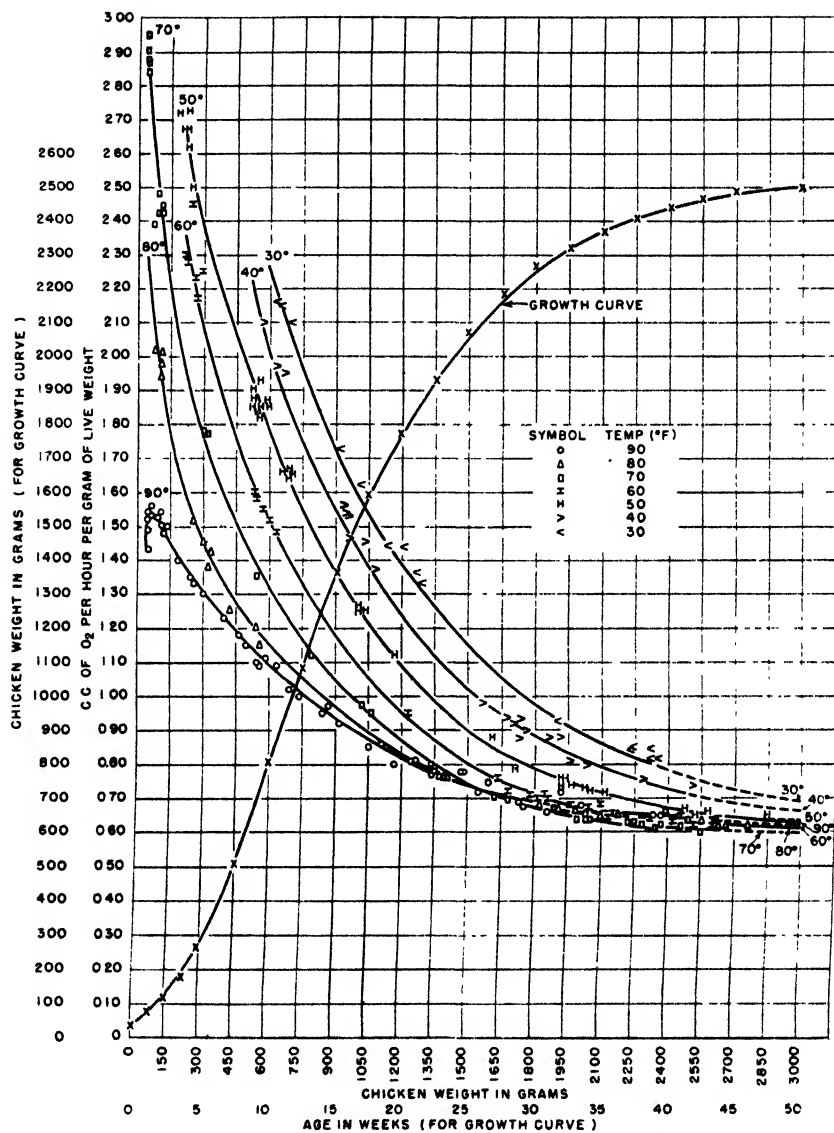


Fig. 2 Mean oxygen consumption from 8 A.M. to 8 P.M. of chickens from hatch to maturity at various environmental temperatures and growth curve. (Source of data for growth Curve: research by poultry section. A. H. Div., B. A. I., Agric. Res. Center, Beltsville, Md.)

tion and the heat elimination for the same groups of chickens as those in figure 3 were determined in the same manner as the oxygen consumption. However, as the type of curve is the same, the curves are not shown but the values are tabulated in tables 1 and 2.

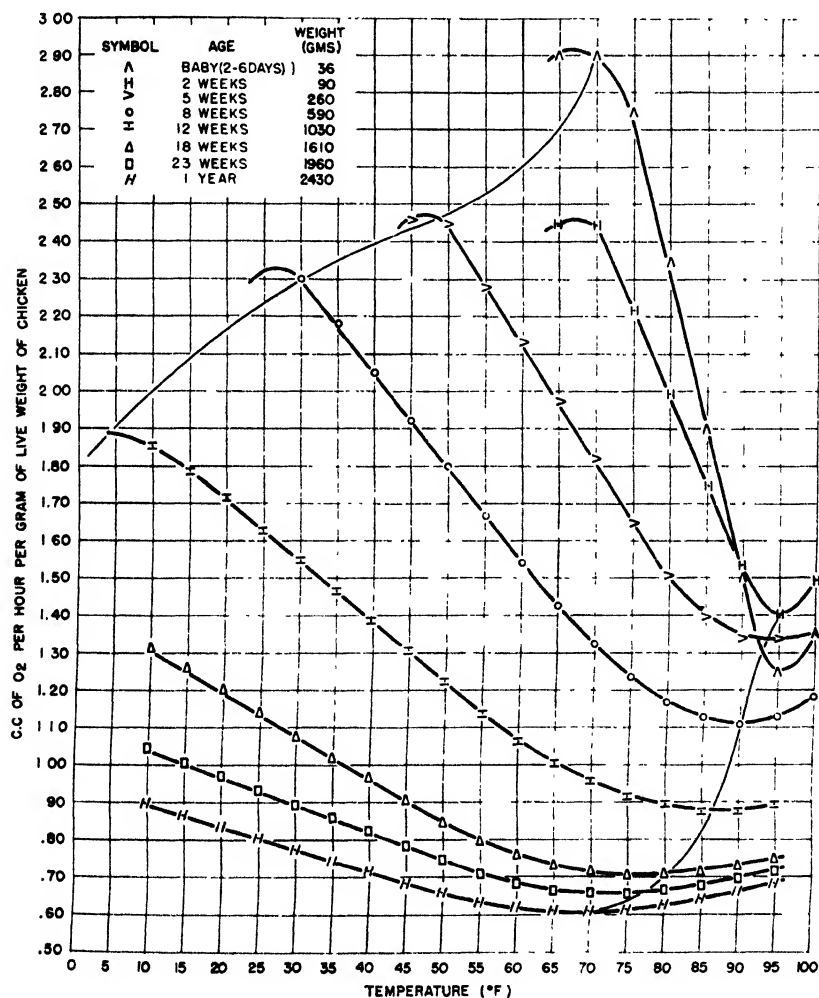


Fig. 3 Oxygen consumption of chickens at various temperatures: mean of 8 A.M. to 8 P.M. values.

The upper temperature limits for survival for 24 hours under the conditions of these experiments lie between 90°F. for mature fowls and 103°F. for baby chicks. The temperature limits become progressively lower than 103°F. as the age of the chick increases. Some hens died during experiments, at

TABLE 1

*Carbon dioxide elimination of chickens at various environmental temperatures.  
Mean values between 8 a.m. and 8 p.m.*

TEMPERATURE	CHICKENS								
	Age	0-1	2	5	8	12	18	23	52 weeks
	Wt.	36	90	260	590	1030	1610	1960	2430 gm.
°F	<i>All values are ml CO<sub>2</sub> per hour per gram live weight</i>								
10						1.34	0.94	0.76	0.64
15						1.28	0.89	0.73	0.62
20						1.22	0.86	0.70	0.60
25					1.67	1.16	0.81	0.68	0.58
30					1.65	1.10	0.77	0.65	0.56
35					1.56	1.04	0.73	0.62	0.54
40					1.47	0.99	0.69	0.59	0.51
45				1.78	1.38	0.93	0.65	0.57	0.49
50				1.75	1.30	0.88	0.61	0.54	0.48
55				1.63	1.20	0.82	0.57	0.51	0.46
60				1.52	1.10	0.77	0.54	0.49	0.45
65	2.08	1.75	1.41	1.02	0.72	0.52	0.48	0.48	0.44
70	2.08	1.75	1.30	0.94	0.69	0.51	0.47	0.47	0.44
75	1.97	1.58	1.25	0.88	0.66	0.51	0.47	0.47	0.44
80	1.67	1.42	1.08	0.83	0.64	0.51	0.48	0.48	0.45
85	1.35	1.26	0.99	0.80	0.64	0.52	0.48	0.48	0.46
90	1.07	1.10	0.96	0.80	0.63	0.53	0.49	0.49	0.47
95	0.90	1.00	0.96	0.82	0.64	0.54	0.51	0.51	0.49
100	1.00	1.08	0.99	0.86	0.66				

90°F., chickens 8 weeks old and older died at 95°F., chickens 2 weeks and 5 weeks old, survived at 100°F., but died at 102°F., while the baby chicks survived at 103°F.

All the curves on figure 3 have the same general form. The metabolic rate is at a minimum at a definite temperature for each age. This temperature is 95°F. for baby chicks and chicks 2 weeks old and as the chicken gets older the temperature at which metabolism is at a minimum is progressively lower,

until for 1-year-old chickens the minimum metabolism occurs at approximately 70°F.

The metabolic rate increases as the temperature is decreased below that at which the minimum metabolism occurs and continues to increase until the chicks are no longer able to produce enough heat to maintain body temperature and perish from the cold. The metabolic rate increases most rapidly for the baby chicks and becomes progressively less and less as the chicken gets older. The rate also increases at temperatures

TABLE 2

*Heat elimination of chickens at various environmental temperatures.  
Mean values between 8 a.m. and 8 p.m.*

TEMP- PERA- TURE	CHICKENS								
	Age	0-1	2	5	8	12	18	23	52 weeks
	Wt.	36	90	260	500	1030	1610	1960	2430 gm.
° F	All values are calories per hour per gram live weight								
40					9.10	6.15	4.25	3.65	3.20
45				11.00	8.55	5.80	4.00	3.50	3.10
50				10.80	8.00	5.45	3.75	3.35	2.95
55				10.30	7.45	5.10	3.55	3.20	2.85
60				9.60	6.80	4.75	3.35	3.10	2.80
65	12.90	10.85	8.90	6.30	4.50	3.25	3.05	2.75	
70	12.90	10.85	8.15	5.85	4.25	3.15	3.00	2.75	
75	12.00	9.90	7.45	5.45	4.10	3.10	2.95	2.75	
80	10.30	8.85	6.70	5.15	4.00	3.10	2.85	2.80	
85	8.40	7.85	6.20	5.00	3.90	3.15	3.00	2.85	
90	6.60	6.80	5.95	4.95	3.70	3.25	3.10	2.90	
95	5.50	6.25	5.95	5.00	3.95	3.40	3.20	3.00	
100	6.00	6.50	6.20						

above that for the minimum metabolic rate. This increase is due primarily to the extra effort entailed in panting to evaporate sufficient water from the respiratory tract for cooling purposes in maintenance of body temperature. The increase in rate continues to a point where the bird can no longer evaporate sufficient water for cooling and perishes from the heat. The great increase in water elimination for the ages studied is shown in figure 4. The flexures at the low temperature end of the curves for baby chicks, and chickens 2 weeks,

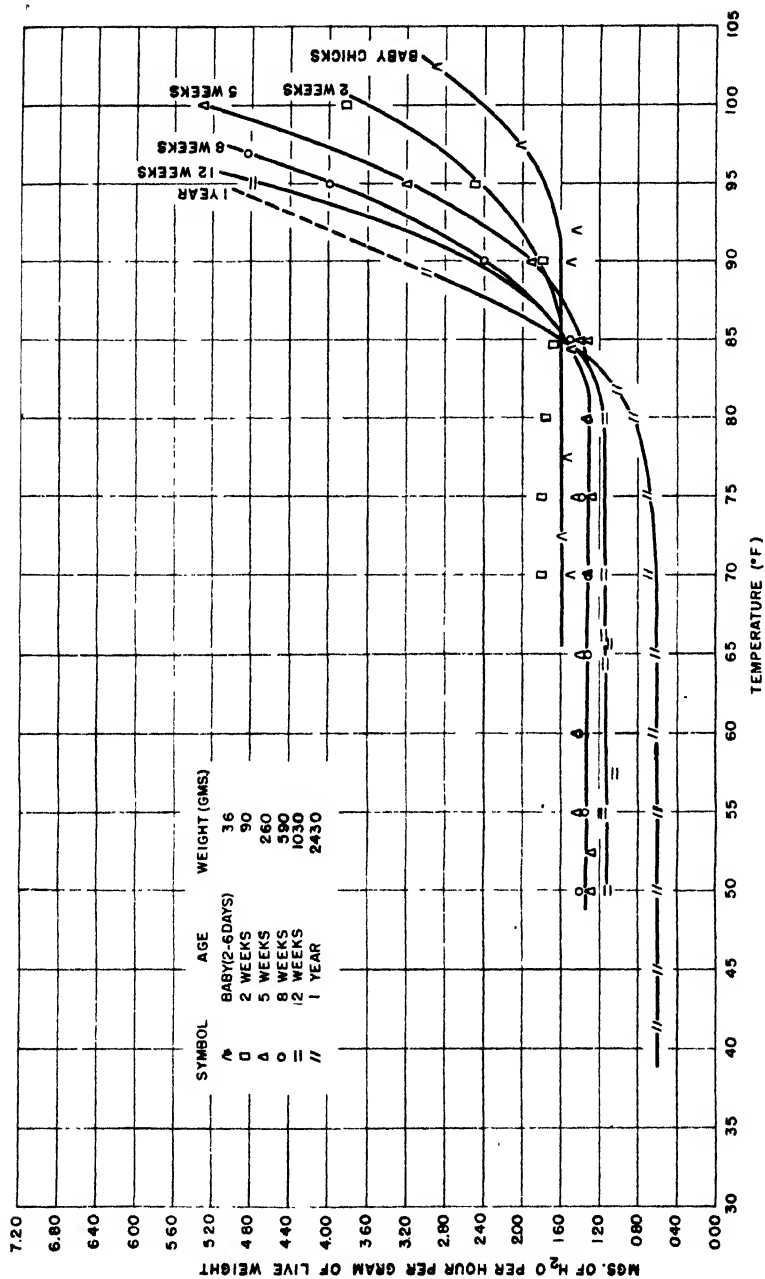


Fig. 4 Water of respiration.

5 weeks and 8 weeks old indicate the lowest temperatures that the chickens could survive for 24 hours without ill effects, under the conditions of the experiment.

The last point plotted on each of these four curves (at 65°F. for baby chicks and chickens 2 weeks old, 45°F. for 5-week-old and 25°F. for 8-week-old chickens) was obtained from results for short intervals of time as the chickens could not stand 24 hours' exposure. The lowest temperature at which chickens older than 8 weeks can survive under similar conditions has not yet been determined. It will be noted that chickens 2 weeks of age were unable to survive a temperature lower than the limit for baby chicks, i.e., 70°F.

*Respiratory and thermal quotients and food  
material metabolized*

The respiratory quotient and the carbon dioxide and oxygen thermal quotients were computed from observed data for the period from 8 A.M. to 8 P.M. There appeared to be very little variation in the respiratory or thermal quotients with either temperature or age of bird. All values of the R.Q. approximated 0.717. The R.Q. did seem to decrease slightly below 40°F., reaching a value of 0.715 at 10°F. Whether this is true or an artifact, it is impossible from the data to state. The oxygen T.Q. was 3.11 and the carbon dioxide T.Q. 3.16.

Equations <sup>1</sup> were set up in a previous paper (Barott et al., '38) for the computation of the relative quantities of carbohydrate, fat and protein being metabolized. Inserting the computed values of the respiratory and thermal quotients in these equations, we found that the relative amounts of the

<sup>1</sup> Inasmuch as direct measurements of both heat and gaseous metabolism were made, the relative quantities of carbohydrate, fat and protein being metabolized could be estimated from the R.Q. and the T.Q.'s. The method of estimation was as follows: If  $x$ ,  $y$  and  $z$  represent, respectively, the percentages of carbohydrate, fat and protein being metabolized, the following equations may be set and readily solved:  $1.000 x + 0.707 y + 0.705 z = 100 \times \text{the observed R.Q.}$ ;  $2.568 x + 3.373 y + 3.110 z = 100 \times \text{the observed CO}_2 \text{ thermal quotient}$ ;  $3.531 x + 3.279 y + 3.018 z = 100 \times \text{the observed O}_2 \text{ thermal quotient}$ .

three food materials metabolized by the chickens in these experiments were: protein, 69%; fat, 27%; and carbohydrate, 4%.

### *Respiratory water elimination*

Some difficulty was encountered in determining the amount of water eliminated by respiration for all chickens 12 weeks old and younger. The fine wire mesh of the cages necessary for baby chicks and those 2 weeks of age prevented the droppings from passing through and although the cages for chickens 5 weeks, 8 weeks, and 12 weeks of age were placed over a pan of oil in which the droppings were collected, some droppings always adhered to the cages. During the course of the experiment, the water in these exposed droppings evaporated and was absorbed with the water of respiration, making it impossible to get a correct figure for the latter. Determinations have shown, however, that the amount of water in the feces is approximately 75% of the total weight. Some tests made at this laboratory showed that the feces contained about 30% of water at the time of removal from the cages. Assuming these values to be correct, one can compute the approximate amount of water evaporated from the feces. Subtracting this amount of water from the total water collected in the absorption flasks, gave an estimate of the water eliminated by respiration.

The results were plotted in figure 4 and curves drawn through the plotted points. The points on the curve for the water of respiration of hens are observed values as the feces were collected under oil and did not introduce an error into the water collected in the flasks. The curves for the younger chickens show the general trend of the respiratory water elimination and although the magnitude of the error is not great, they are not to be considered as being of the same accuracy as the curve for hens.

No value for water elimination could be obtained below 40°F., because the precipitation of moisture on the cooling unit.

The water of respiration for each age studied remains fairly constant at all temperatures up to the temperature of minimum metabolism. Above this temperature it becomes necessary for the chicken to exhale an additional amount of water for the purpose of cooling. This amount rapidly becomes greater and greater as the temperature is increased. The water respired per gram weight of chicken, at temperatures below the minimum, is lowest for hens and becomes higher for the younger chickens. As previously stated, the data for chickens 12 weeks old and younger are only fairly accurate estimates and not true measurements as in the case of the hens.

#### SUMMARY AND CONCLUSIONS

The energy and gaseous metabolism of Rhode Island Red female chickens, averaging 4 days, 2 weeks, 5 weeks, 8 weeks, 12 weeks, 18 weeks, 23 weeks and 1 year old, were determined by use of one of the respiration calorimeters in the calorimetry laboratory of the Bureau of Animal Industry, Agricultural Research Center, Beltsville, Maryland. The instrument is similar to the one described by Barott ('37). More than 500 1-day experiments were made.

Results were obtained with environmental temperatures at approximately 5°F. intervals in the range from 20° to 103°F. Conditions other than temperature were: Relative humidity 50–60%, oxygen content 21%, and carbon dioxide content less than 1%.

The oxygen consumption was measured for each 2-hour period, and the heat, water and carbon dioxide elimination for each 4-hour period. The results define the metabolic rate of each age of chicken studied, at each environmental temperature investigated. A point of flexure occurs at the temperature where metabolism is a minimum. This minimum occurs at 95°F. for baby chicks and chickens 2 weeks old. As the chicken gets older the minimum occurs at a temperature which is progressively lower until for 1-year-old hens, the minimum occurs at approximately 70°F. The maximum meta-



bolism occurs at the lowest temperature the chicken could survive. This temperature has not yet been determined for chickens older than 8 weeks.

The data obtained for each 24-hour period show the typical diurnal rhythm in the metabolism of the chicken, with a maximum value at 8 A.M. and a minimum value at 8 P. M.

The amounts of protein, fat and carbohydrate metabolized by the chickens were computed. The amounts of the three food constituents metabolized were: protein, 69%; fat, 27% and carbohydrate, 4%.

The observed R.Q. approximates 0.717 at all ages and all temperatures. The observed oxygen T.Q. is 3.11 and the observed carbon dioxide T.Q. is 3.16.

The water of respiration is fairly constant at temperatures below that where minimum metabolism occurs. Above this temperature there is an enormous increase due to the large amount of water exhaled for cooling at the higher temperatures.

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# THE THIAMINE, RIBOFLAVIN, NICOTINIC ACID AND PANTOTHENIC ACID CONTENT OF COLOSTRUM AND MILK OF THE COW AND EWE

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In most mammalian species the new-born under natural conditions depends for varying periods of time almost entirely on the secretion from the mammary gland for food. Colostrum differs in composition from milk in several respects. While extensive studies have been made of the carotene and vitamin A content of colostrum of the cow (Daun, '33; Henry, Houston and Kon, '40), sheep (Underwood and Curnow, '44) and human (Daun, '36) information on the B vitamins in colostrum is meagre. The report by Lundquist and Phillips ('43) on the possible role of some of the vitamins in the prevention of certain diseases of the new-born calf prompted the authors to make a study of the amounts of thiamine, riboflavin, nicotinic acid and pantothenic acid in colostrum as compared with milk. Information on the B vitamins in colostrum affords a basis for the preparation of substitutes in feeding new-born animals, and especially for the first few days of life which is a critical period.

The amount of thiamine in human milk is definitely influenced by the dietary intake (Knott et al., '43) and a similar relationship probably holds for the other B vitamins in colostrum and milk of species for which they are a dietary

essential. It was felt that this variable could be largely eliminated or reduced to a minimum if the studies were made with ruminants as the B vitamins are not dietary essentials for them.

## EXPERIMENTAL

### *Methods*

Samples of colostrum were obtained from cows on the day of parturition. Preliminary studies made on colostrum collected at frequent intervals after parturition showed that there was no essential change in any of the four vitamins under study during the first 24 hours. A sample of milk was collected from each of the cows after they had been in milk for 30 days or longer. The two breeds represented are the Holstein and Jersey.

The colostrum was collected from the ewes on the day of parturition. The samples of milk were not from the same ewes as the colostrum, but it is presumed that they would be representative for the species.

Precautions were taken to protect the samples against photochemical destruction of riboflavin. Thiamine was determined by the thiochrome method of Hennessy ('42). Riboflavin was determined by the microbiological method of Snell and Strong ('39) on the autoclaved sample following filtration at a pH of approximately 4.6 to remove foreign growth stimulants. Nicotinic acid was determined by the microbiological method of Krehl, Strong and Elvehjem ('43) with the slight modification devised by Pearson and Luecke ('45) which was found to be satisfactory for colostrum and milk. Pantothenic acid was determined by the method of Neal and Strong ('43) following the enzymatic liberation of the vitamin by takadiastase and papain.

## RESULTS

The mean values and standard deviations for thiamine, riboflavin, nicotinic acid and pantothenic acid for colostrum and

milk of the cow and ewe are shown in table 1. There is a distinct species difference in the amount of riboflavin, thiamine and nicotinic acid in both the colostrum and milk. The colostrum of the ewe is approximately 74% higher in thiamine than the colostrum of the cow while for milk the corresponding difference is around 58%. The difference for riboflavin is more striking as the amount in the colostrum of the ewe is about 3.3 fold greater than in the colostrum of the cow while the corresponding figure for milk is about 2.5 fold. There is a similar relationship for nicotinic acid which is higher in

TABLE 1  
*Thiamine, riboflavin, nicotinic acid and pantothenic acid  
content of colostrum and milk.  
(Values in  $\mu\text{g. per ml.}$ )*

VITAMIN	No. of animals	C O W				E W E					
		Colostrum		Milk		No. of animals	Colostrum		Milk		
		Mean	S. D. <sup>1</sup>	Mean	S. D. <sup>1</sup>		Mean	S. D. <sup>1</sup>	Mean	S. D. <sup>1</sup>	
Thiamine	25	0.62	0.06	0.38	0.05	5	1.08	0.31	0.60	0.12 <sup>2</sup>	
Riboflavin	32	6.10	1.85	1.77	0.31	14	20.08	5.46	4.36	0.66	
Nicotinic acid	32	0.96	0.29	0.91	0.16	14	1.97	0.44	3.93	1.09	
Pantothenic acid	32	2.24	0.87	3.67	0.57	14	2.62	0.71	3.66	0.79	

<sup>1</sup> Standard deviation.

<sup>2</sup> Twelve samples of milk.

both the colostrum and milk of the ewe than in cow's colostrum and milk. There is an interesting relationship between the rates of gain of the new-born and the amount of thiamine, riboflavin and nicotinic acid in the milk of the two species. The new-born lamb doubles its weight in approximately 14 days while the calf requires around 47 days to double its weight. One can postulate that the higher amounts of thiamine, riboflavin, and nicotinic acid in the colostrum and milk of the ewe constitute one of the functions of the more rapid growth of the new-born of this species. A limited number of observations in this laboratory has revealed that the amounts of these three vitamins in the milk of the human and horse is much lower than in cow's milk. The rate of growth of the

new-born of these species is likewise much slower than that of the new-born calf.

There are definite differences in the amounts of the vitamins in the colostrum and milk of both species. The difference between the mean values for milk and for colostrum has been tested by Fisher's *t* value. The difference in the values of colostrum and milk for riboflavin and pantothenic acid is highly significant for both species. The difference between the amount of thiamine in the colostrum and milk of the cow is highly significant, and for the ewe is significant. The difference in the amount of nicotinic acid in the colostrum and milk of the ewe is highly significant, but for the cow there was no significant difference.

The colostrum of the cow contained an average of 0.62  $\mu\text{g.}$  of thiamine per milliliter while the milk contained 0.38  $\mu\text{g.}$  per milliliter. The corresponding values for the colostrum and milk of the ewe are 1.08  $\mu\text{g.}$  and 0.60  $\mu\text{g.}$  per milliliter, respectively. The higher thiamine content of colostrum of both the cow and ewe differ from the findings of Knott et al. ('43) on human colostrum and milk. These workers reported that human colostrum contained almost no thiamine and that there was a gradual increase in the amount up to 3 weeks after parturition. In contradistinction to this Escudero and Sola ('43) reported that human colostrum contained 43% more thiamine than human milk. A possible explanation of these conflicting reports is that the pre-parturient dietary regimen of the subjects may have furnished quite different amounts of thiamine.

The colostrum of the cow contained an average of 6.10  $\mu\text{g.}$  of riboflavin per milliliter while milk contained 1.77  $\mu\text{g.}$  per milliliter which is a ratio of about 3.4 to 1. The values for riboflavin in the colostrum and milk of the ewe are 20.08  $\mu\text{g.}$  and 4.36  $\mu\text{g.}$  per milliliter, respectively. The higher riboflavin content of colostrum in these species is in accord with the higher value observed in human colostrum as compared with human milk by Escudero and Sola ('43).

The nicotinic acid content of colostrum and milk of the cow is not significantly different. However, the milk of the ewe

contains almost twice as much nicotinic acid as the colostrum. This is the only respect in which the general relationship between the amount of each vitamin in the colostrum and milk of the cow differs from that in the colostrum and milk of the ewe.

The milk of both the cow and ewe contains significantly more pantothenic acid than the colostrum of the respective species. The higher pantothenic acid content of milk and the higher nicotinic acid content of the milk of the ewe is in contrast with

TABLE 2

*Effect of progress of lactation period on level of thiamine, riboflavin and pantothenic acid in cow's colostrum and milk.*

DAYS AFTER PARTURITION	THIAMINE	RIBOFLAVIN	PANTOTHENIC ACID
	$\mu g / ml.$	$\mu g / ml.$	$\mu g / ml.$
0	0.58	5.69	1.73
1	0.59	3.53	3.20
2	0.59	2.67	3.96
3	0.59	2.32	4.24
4	0.58	2.03	4.01
5	0.59	2.03	4.05
6	0.58	1.93	4.19
7	0.57	1.87	4.29
8	0.56	1.87	4.38
9	0.56	1.89	4.16
30	0.38	1.83	3.82

thiamine and riboflavin which are higher in colostrum. The values reported here for the thiamine, riboflavin, nicotinic acid and pantothenic acid in cow's milk agree fairly well with the figures in the literature. They were included in the present study as they afford a more accurate control based on colostrum and milk samples from the same animals.

The daily change in the amount of thiamine, riboflavin and pantothenic acid in colostrum of the cow following parturition is shown in table 2. The data for nicotinic acid have been omitted from this table as there was no significant difference in the amount in cow's colostrum and milk. Samples were

collected the day of parturition and daily thereafter for 9 successive days. A sample of milk was collected again about the thirtieth day. The data in table 2 are average values for eight animals.

The average thiamine value of the colostrum for the eight cows the day of parturition was 0.58  $\mu\text{g.}$  per milliliter and 9 days later it was essentially the same being 0.56  $\mu\text{g.}$  per milliliter. The average thiamine content of the milk of the eight cows 30 days after parturition was 0.38  $\mu\text{g.}$  per milliliter which is the same as that shown for milk in table 1. It is interesting that the high thiamine content of colostrum and subsequently of the milk persists for a much longer period than does the high riboflavin level. The riboflavin declines rapidly after the first day and approaches the normal value for milk by about the sixth or seventh day of lactation.

There is a marked increment in the pantothenic acid content of the colostrum as evidenced by the change from 1.73  $\mu\text{g.}$  per milliliter the day of parturition to 3.20  $\mu\text{g.}$  per milliliter on the second day. The pantothenic acid level approaches the normal level for milk about the third day following parturition. No particular significance is attached to the slightly higher levels observed a few days later.

#### SUMMARY

There is a definite species difference in the amounts of thiamine, riboflavin and nicotinic acid in both the colostrum and milk of the cow and ewe. The colostrum and milk of the ewe are both much richer in each of these vitamins than the colostrum and milk of the cow.

The colostrum of both the cow and ewe is much higher in thiamine and riboflavin than is the milk of the respective species. The colostrum of the cow contains an average of 0.62  $\mu\text{g.}$  of thiamine and 6.10  $\mu\text{g.}$  of riboflavin per milliliter while the corresponding values for the milk are 0.38  $\mu\text{g.}$  and 1.77  $\mu\text{g.}$  per milliliter. Ewe's colostrum contains 1.08  $\mu\text{g.}$  of thiamine and 20.08  $\mu\text{g.}$  of riboflavin per milliliter while the corresponding values for the milk of the ewe are 0.60  $\mu\text{g.}$  and 4.36  $\mu\text{g.}$  per

milliliter. There was no significant difference in the nicotinic acid content of cow's colostrum and milk, but ewe's milk contained almost twice as much as the colostrum. The pantothenic acid level of the milk of both species is higher than it is in the colostrum.

The riboflavin and pantothenic acid for the cow attain the level for normal milk within less than a week after parturition, but the high level for thiamine persists beyond the tenth day.

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# VEGETABLE CROPS IN RELATION TO SOIL FERTILITY

## IV. NUTRITIONAL VALUES OF NEW ZEALAND SPINACH <sup>1</sup>

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There is still a paucity of information on the question as to just how much green leafy vegetables contribute to human nutrition. Recently, they have been termed "protective" foods because of their dietary importance as sources of calcium and vitamins A, C, and G, which are among the nutrients most apt to be deficient in American dietaries (Report of the Committee on Diagnosis and Pathology of Nutritional Deficiencies Food and Nutrition, '43). However, with respect to calcium, members of the Goosefoot family of greens (spinach, Swiss chard, beet greens) and New Zealand spinach may contain sufficient oxalic acid to entirely nullify this nutrient contribution, as well as to render unavailable in the diet additional quantities of calcium in other foods such as milk (Kohman, '34, '39; Fincke and Sherman, '35; Fairbanks and Mitchell, '38; Tisdall and Drake, '38; Spiers, '39).

According to one report (Kohman, '34), New Zealand spinach contained an unusually high percentage of oxalic acid, its concentration being about equal to the toxic amounts in rhubarb leaves. Also, its vitamin C content has been listed at a relatively low figure (Hewston and Marsh, '42). Since this crop

<sup>1</sup> A contribution by the Department of Horticulture, Missouri Agricultural Experiment Station Journal Series no. 984.

is so universally recommended as a desirable vegetable and as a good substitute for some other greens which cannot be grown so successfully during hot weather, additional information on its nutritional values, as compared with other leafy vegetables, was felt essential. Moreover, the effects of the soil and its fertility level on the nutrient quality of a given crop may alter its "protective" value, and are, therefore, commanding an increased attention in experimental studies with vegetables.

#### EXPERIMENTAL

New Zealand spinach was grown under controlled greenhouse conditions in gallon glazed crocks, using colloidal clay cultures as a soil medium (Albrecht and Schroeder, '39). A series of sixteen treatments was prepared by supplying calcium and nitrogen at levels of 5, 10, 20, and 40 milliequivalents (m.e.) with all possible (i.e. 16) combinations of these amounts of the two nutrients. Thus, for each level of calcium, there were four levels of nitrogen, and for each level of nitrogen, there were four of calcium. Each of the sixteen series was replicated ten times with two plants in each replication.

The calcium and nitrogen were provided by adding variable quantities of calcium acetate and ammonium nitrate, respectively, to the correspondingly required clay aliquots, replacing the naturally adsorbed hydrogen thereon with cationic nutrients. Other materials supplied, but in constant amounts for all treatments, consisted of 20 m.e. each of potassium and phosphorus and 6 m.e. of magnesium and sulfate.

The plants were grown for a period of about 70 days and the tops harvested, at which time they were judged to be in the most desirable eating condition. One specimen from each replication, making a total of ten individual plants separately analyzed in each treatment, was utilized for vitamin C analyses, conducted according to methods described by Wittmer, Schroeder and Albrecht ('45). The remaining ten plants from each treatment were harvested, dried, ground, and used collectively for the chemical determinations of oxalate, total

nitrogen, phosphorus, calcium, and magnesium. The A.O.A.C. ('40) methods for calcium and magnesium were used. Oxalate was measured according to Pucher, Wakeman and Vickery ('41), total nitrogen according to Murneek and Heinze ('37) and phosphorus according to Fiske and SubbaRow ('25).

### RESULTS

The total growth of the plants and their composition, as related to increments of calcium and nitrogen, are given in table 1. The treatments are arranged into four nitrogen groups, in which the different calcium levels are averaged to show the influence of variable nitrogen. Regrouping, according to the calcium applied, is possible if the rather insignificant effects of calcium are desired.

The first outstanding feature observed with reference to the crop's behavior was its very decided response to nitrogen as a fertilizer salt. This influence manifested itself in both the quantity and quality of vegetation produced. Yields were a direct function (treatment 5 a possible exception) of the supply of nitrogen, almost without regard to the soil calcium supply. Within the edible portions of the plant, the vitamin C content was significantly depressed, as a result of increased nitrogen fertilization, which is in accord with recent reports on other fruits and vegetables (Jones et al., '44; Hamner, Bernstein and Maynard, '45; Wittwer, Schroeder and Albrecht, '45). Also, as more nitrogen was applied, decreases similar to those for vitamin C occurred in the concentrations of oxalate and phosphorus, though, as might be expected, the plants' total nitrogen percentages increased.

Of perhaps even more importance in human nutrition is the fact that New Zealand spinach is a very lean source for some of the essential food items, normally thought to be plentifully supplied in the diet by all green leafy vegetables. The concentration range for vitamin C, in milligrams per 100 gm. of fresh weight, was  $20.9 \pm 0.48$  for high nitrogen plants to  $29.6 \pm 0.44$  for those more deficient in this element (table 1). These values, when listed with those for other com-

TABLE 1  
*Variations in yield, vitamin C, oxalate, and minerals of New Zealand spinach grown under variable levels of nitrogen and calcium.<sup>1</sup>*

TREATMENT	APPLIED		YIELD PER 10 PLANTS		VITAMIN C per 100 gm.	OXALATE		MINERAL ANALYSES				Mg	
	N	Ca	Fresh wt	Dry wt.		%	%	N	PO <sub>4</sub>	C <sup>a</sup>	%	Mg	%
	m c.	m c.	gm.	gm.	mgm.			%	%	%			
	N group I												
1	40	40	467.6	27.21	20.3 ± 0.70	6.07	6.12	1.63	0.460			0.554	
5	40	20	280.0	16.58	18.9 ± 0.98	5.80	5.97	1.65	0.659			0.164	
9	40	10	461.2	28.92	21.4 ± 1.01	6.05	6.07	1.73	0.456			0.262	
13	40	5	466.2	29.88	22.8 ± 0.66	6.17	6.18	1.67	0.548			0.041	
Average			418.8	25.65	20.9 ± 0.48	6.02	6.09	1.67	0.531			0.255	
	N group II												
2	20	40	422.2	25.80	28.5 ± 0.87	7.08	4.79	2.34	0.536			0.393	
6	20	20	367.7	22.17	27.9 ± 1.18	7.42	5.25	2.21	0.479			0.086	
10	20	10	325.1	20.48	25.3 ± 1.54	7.13	5.60	1.78	0.602			0.337	
14	20	5	392.1	24.90	26.9 ± 1.06	6.86	5.76	2.06	0.528			0.025	
Average			376.8	23.34	27.2 ± 0.59	7.12	5.35	2.10	0.536			0.211	
	N group III												
3	10	40	267.7	17.37	29.5 ± 0.63	7.95	3.58	3.68	0.570			0.343	
7	10	20	230.8	14.73	28.7 ± 0.74	7.52	4.64	2.97	0.548			0.069	
11	10	10	219.4	14.83	29.5 ± 0.57	7.24	4.76	2.52	0.520			0.389	
15	10	5	158.5	11.29	30.5 ± 1.38	6.77	5.06	3.25	0.506			0.074	
Average			219.1	14.56	29.6 ± 0.44	7.37	4.51	3.11	0.536			0.219	
	N group IV												
4	5	40	142.2	9.40	27.0 ± 0.72	9.36	3.06	3.63	0.618			0.368	
8	5	20	122.8	8.42	28.4 ± 1.02	7.34	3.33	4.00	0.634			0.340	
12	5	10	121.5	8.43	30.0 ± 1.16	8.57	3.84	3.56	0.583			0.131	
16	5	5	98.8	7.24	29.6 ± 0.80	7.42	3.82	4.40	0.514			0.324	
Average			121.3	8.37	28.7 ± 0.49	8.17	3.51	3.90	0.587			0.291	

<sup>1</sup> Vitamin C given in milligrams per 100 gm. fresh weight, oxalate and mineral analyses in percentage of dry weight.

mon greens, are very low. Members of the Goosefoot family (spinach, Swiss chard, beet greens) have a range of concentrations from about 40 to 100 mg., while for vegetables of the Mustard family (kale, turnip greens, collards, mustard greens, broccoli) the range is from about 75 to 200.

An especially deleterious feature of the composition of New Zealand spinach is its high oxalate content. Oxalic acid in chemical combination with calcium forms a very insoluble compound. This is likely to be true also of magnesium (Pierce and Appleman, '43). Since New Zealand spinach has been reported to contain an unusually high percentage of oxalic acid, sufficient, perhaps, to more than precipitate and form insoluble, indigestible oxalates with the entire supply of the plant's calcium and magnesium, the degree to which the oxalate would balance stoichiometrically and make insoluble these two basic minerals in the plant may be determined by converting to milliequivalents the percentages of oxalate, calcium and magnesium given in table 1.

On such a chemically equivalent basis, the oxalate would exceed by several times the calcium at all fertility levels. Also, the total oxalate would be far in excess of the plant's calcium and magnesium combined. Thus is left a sizable surplus of soluble oxalates for possible combination with calcium derived from other foods in the diet. If enough calcium from other sources such as milk were to be added to balance the excess oxalate, it would require from about four to six times as much as the crop itself contributes. With the problems of calcium availability in New Zealand spinach, there are involved not only initially larger quantities of oxalate, but also amounts of calcium and magnesium native in the crop, which are considerably lower than those in any related vegetables (Schroeder and Albrecht, '42; Wittwer, Albrecht and Goff, '45).

#### SUMMARY AND CONCLUSIONS

New Zealand spinach, compared with other leafy vegetables as a "protective" food, is a poor source for vitamin C.

Its low calcium and magnesium concentrations with an extremely high oxalate content suggest a negative mineral contribution; there is likely a precipitation of quantities of calcium derived from other foods consumed, tantamount to from four to six times that furnished by the plant itself.

The response of the crop to nitrogen fertilization was (1) practically independent of the soil calcium, and (2) reflected by an increased yield of vegetation which generally became progressively inferior in nutritional value with each increment of nitrogen.

New Zealand spinach is at the bottom of the list of green leafy vegetables insofar as its vitamin C and calcium contributions are concerned.

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# THE RELATION OF VITAMIN A INTAKE TO LENGTH OF LIFE, GROWTH, TOOTH STRUCTURE AND EYE CONDITION

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THREE FIGURES

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## INTRODUCTION

The minimum vitamin A requirement has been defined by Smith ('42) as the amount of vitamin A calculated to a daily dose that just prevents clinical symptoms of vitamin A deficiency. This amount has been found adequate for good growth and general well-being but permits little or no storage. Goss and Guilbert ('39) on the basis of data obtained from vaginal smear records estimate the minimum vitamin A requirement for the albino rat at 1.8-2.2 units of vitamin A per 100 gm. body weight. This amount of vitamin A was said to be sufficient for normal growth.

Lewis et al. ('42) have shown that intakes up to 25 U.S.P. units of vitamin A per rat daily result in improved growth. There was no increase in growth when amounts greater than 25 units daily were fed. Baumann, Riising and Steenbock ('34) found that as little as one International Unit (I.U.) of vitamin A per rat daily was sufficient to cure xerophthalmia and restore growth but that 7 to 17 I.U. were required to produce storage in the liver. Horton et al. ('41) conclude from their work that about 20 I.U. daily per rat were sufficient to main-

tain the blood level of vitamin A but were not sufficient for storage.

Irving and Richards ('39) have postulated that the vitamin A requirement of the rat increases with age, 3 I.U. daily being sufficient to maintain normal colored teeth in the rat up to 52 days of age. However, at 180 days of age, the teeth were not entirely normal in the animals receiving 3 I.U. daily.

Guilbert and Hart ('35) believe that vitamin A requirements are a function of the body weight. The majority of experiments reported in the scientific literature were conducted without consideration of the increasing body weight of the rat. Therefore, the rat fed on a straight daily dose basis would receive less vitamin A per unit of body weight as his size increased.

McCay, Crowell and Maynard ('35) have shown that the life span of retarded growth animals exceeds that of animals which have grown to maturity rapidly. These results were obtained with animals whose growth was retarded by a deficiency of calories. That slower growth due to suboptimal vitamin A levels is not compatible with maximum life expectancy is demonstrated by the experiments of Sherman and MacLeod ('25). These experiments, however, do not provide data on the quantitative vitamin A requirement of the albino rat for maximum life expectancy.

In view of the varying data it seemed advisable to carry on further investigations on the vitamin A requirement of the rat, administering the vitamin A on a body weight basis and carrying the animals throughout the entire life span on the experimental diets. Experiments were designed with the following questions in mind:

1. How does the minimum vitamin A requirement as previously determined compare with the vitamin A requirement for maximum length of life?

2. Is the minimum level of vitamin A previously established from vaginal smear records sufficient for normal growth, normal tooth structure and for normal eyes?

## EXPERIMENTAL PROCEDURE

Young rats 28 days of age were placed on the U.S.P. XII vitamin A-free diet until signs of vitamin A deficiency such as loss in weight and xerophthalmia occurred. They were then assembled into four groups as evenly as possible with regard to age, weight, sex, tooth condition, and general condition. The four groups then received in addition to the vitamin A-free diet the following vitamin A supplements per 100 gm. body weight daily: group A—1 U.S.P. unit, group B—2 U.S.P. units, group C—4 U.S.P. units, group D—20 U.S.P. units. In terms of the requirement postulated by Goss and Guilbert ('39) this would be, respectively,  $\frac{1}{2}$ , 1, 2, and 10 times the minimum.

The vitamin A-free diet had the following percentage composition: casein<sup>1</sup> (vitamin-free), 18.0; starch, 65.0; yeast—dried,<sup>2</sup> 7.5; irradiated yeast,<sup>3</sup> 0.5; salt mixture (U.S.P. no. 1), 4.0; and cottonseed oil, 5.0.

The source of vitamin A was distilled A ester<sup>4</sup> of approximately 300,000 U.S.P. vitamin A units per gram. The same refrigerated stock of vitamin A was used throughout the experiment. The vitamin A potency was determined spectrographically and confirmed by bioassay. Dilutions were freshly made each week and administered orally by syringe. The original stock solution was rechecked from time to time during the experiment to insure its maintenance of potency.

The animals were weighed weekly and the size of the dose adjusted for the next week on the basis of this weight. Records of the appearance of the teeth and general condition of the animals were made at monthly intervals. All animals were maintained on this general regime for the entire life span.

After starting vitamin A supplements the teeth of the animals in groups A, B, C, and D were examined at monthly intervals. Records were kept by colored drawings of the upper

<sup>1</sup> Labco Casein—The Casein Company of America, Inc., New York City.

<sup>2</sup> Strain G—Anheuser-Busch, Inc., St. Louis, Missouri.

<sup>3</sup> Type 9-F—Standard Brands, Inc., New York City.

<sup>4</sup> Distillation Products, Inc., Rochester, New York.

incisor teeth of each animal. Observations were made with respect to (a) chalky appearance, (b) light yellow or white color (i.e., lack of normal orange pigmentation), (c) black discoloration and (d) any combination of these abnormalities. An arbitrary system of grading the abnormalities was adopted. Incisor teeth exhibiting a deep orange pigmentation and a translucent appearance were considered normal and graded 0. Chalky white teeth with black pigmentation were graded 4. Intermediate degrees of abnormality were graded 1, 2, or 3 depending on the estimated severity of the condition. These gradings may not be entirely indicative of degree since there was no way of knowing whether black discoloration or lack of pigment constituted the more severe change.

The eye condition of the animals in all groups was noted every 2 weeks with regard to presence or absence of water, red exudate, corneal ulcers, swelling, etc. In judging the eye conditions an opaque cornea due to a healed corneal ulcer was not considered abnormal since it represented a healing of the abnormal condition even though the eye could not be returned to normal.

It was believed that the cottonseed oil in the diet would provide sufficient vitamin E. However, as the experiment progressed increasing evidence on the effects of vitamin E made it seem desirable to determine this so group C was subdivided when the experiment had been in progress about 1 year. One-half the animals of group C were continued on the original supplement of vitamin A (4 U.S.P. units per 100 gm. body weight). The remainder of the group received, in addition to this dose of vitamin A, 0.1 mg. of mixed tocopherols (from mixed tocopherol<sup>4</sup> solution) daily per 100 gm. of body weight. This is well above the minimal dose of vitamin E established by Mason ('40) for the albino rat. Group C was chosen for the illustration of any possible synergistic effect of added vitamin E since this was proving to be a borderline group. The animals were surviving well, growth was fair, and the general appearance was fair. The teeth were showing

some abnormalities and in general the animals were inferior to group D.

### RESULTS

The data on the effect of the addition of vitamin E to group C after 1 year on the experimental diet are presented in table 1. Apparently the additional vitamin E had no beneficial effect on the tooth structure, the animals in both groups showing about the same increasing severity of abnormalities. Although both the average age at death and the average maximum weights attained were slightly greater for the animals receiving vitamin E, inspection of the probable errors for

TABLE 1  
*Effect of added vitamin E on group C.*

SUB-GROUP	NO ANIMALS	VIT. A PER 100 GM BODY WEIGHT DAILY	ADDED TOCOPHEROLS PER 100 GM. BODY WEIGHT	TOOTH CONDITION		AVE. MAX WEIGHT ATTAINED	AVE AGE AT DEATH
				Start of E therapy	5 mo. later		
		U S P units	mg			gm	days
C <sub>0</sub>	8	4	0	1.9	2.7	334 ± 19	589 ± 24
C <sub>e</sub>	7	4	0.1	1.3	2.2	356 ± 19	629 ± 28

these two values indicates that these differences are not significant. The above data indicate that the basal diet was not improved by additional vitamin E. We have therefore dealt with group C as a single group in considering the rest of the data.

For convenience the results of increasing vitamin A intake for the four groups are discussed under the headings of Longevity, Growth, Teeth, and Eyes.

*Longevity.* The data of table 2 show that the average age at death of the animals increases with the amount of vitamin A fed. The average age at death of thirty-five similarly treated animals on the same diet receiving no vitamin A and serving as negative controls in routine assays was  $69 \pm 0.9$  days. One U.S.P. unit of vitamin A although insufficient for maintenance of life over a long period does increase the life span significantly (i.e., from  $69 \pm 0.9$  to  $80 \pm 2$  days).

The animals in group D receiving the highest level of vitamin A show an average age at death (649 days) which is comparable to that obtained by other workers using synthetic diets. McCay, Crowell and Maynard ('35) report an average age at death for both sexes of 677 days in their fast-growing group.

In figure 1, the mortality data for the four groups are charted at 100-day intervals along with the body weight curves. Inspection of these data reveals that differences in life span between the 4- and 20-unit levels only became apparent after

TABLE 2

*Average age at death of animals receiving varying amounts of vitamin A.*

GROUP	VITAMIN A PER 100 GM BODY WEIGHT DAILY	NO. ANIMALS STARTED		AVE AGE AT DEATH		AVE. AGE AT DEATH ALL ANIMALS
		♂	♀	♂	♀	
	<i>U S P units</i>			<i>days</i>		<i>days</i>
A	1	9	14	81	79	80 ± 2
B	2	9	10	204	260	234 ± 18
C	4	10	10	478	564	521 ± 28
D	20	10	10	622	675	649 ± 30

the animals are 200 to 250 days of age. In other words the higher vitamin A intake was actually effective in prolonging the latter half of the life span and not in reducing mortality during the early part of life. It might be postulated that there is an increased requirement for vitamin A at this time although more data would be required to substantiate this conclusion. Irving and Richards ('39) have suggested that the vitamin A requirement of the rat increases with age. However, in their experiments the increasing body weight of the rat was not compensated for by increasing vitamin A intake.

*Growth.* The growth curves for the four groups of rats during the experiment are shown in figure 1. It is obvious that 1 U.S.P. unit of vitamin A per 100 gm. body weight is insufficient for prolonged maintenance of life or growth in the albino rat. Moderate growth was obtained at the 2-unit level (group

B) and growth was progressively better at the 4- and 20-unit levels.

*Teeth.* The effect of vitamin A deficiency on the structure of the tooth of the albino rat has been described in detail by Wolbach and Howe ('33) and by Schour et al. ('41). Irving and Richards ('39) have found 3 I.U. of vitamin A daily insufficient to maintain entirely normal teeth in older animals.

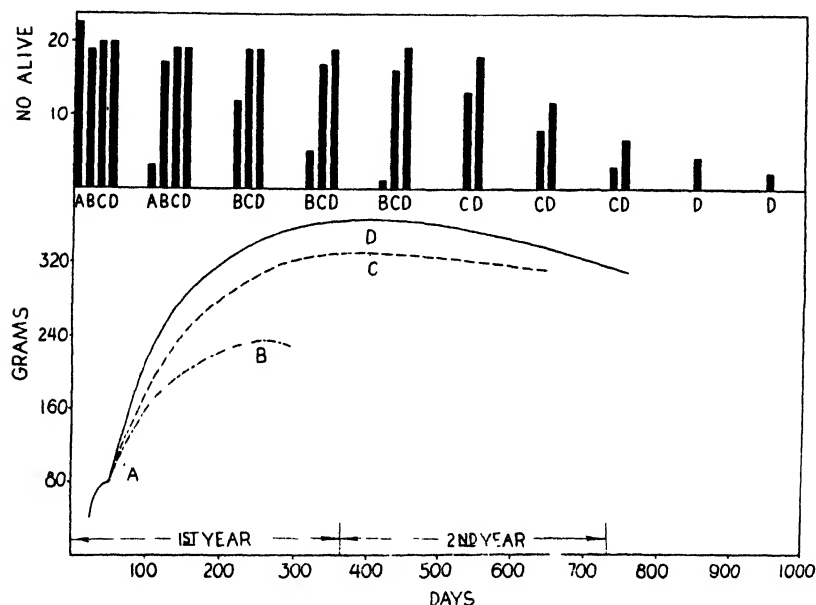


Fig. 1 Body weight curves of rats receiving different amounts of vitamin A per 100 gm. of body weight. The columns at the top show the number alive in each group at 100-day intervals. A—1 U.S.P. unit, B—2 U.S.P. units, C—4 U.S.P. units, D—20 U.S.P. units.

In our experiments the incisor teeth became uniformly chalky (opaque) during the preliminary depletion period and the orange pigmentation changed from slightly lighter to nearly white in some cases. Frequently a black mottling of the teeth was observed. We note that other workers have observed the chalkiness and the lack of pigmentation but we have seen no mention in the literature of a black discoloration



accompanying vitamin A deficiency. This black discoloration appeared frequently in groups A, B, and C of this experiment but was of rare occurrence in group D. We have made no detailed histological studies of the discoloration. The symptom also appears frequently in animals depleted for vitamin A assays in this laboratory. It does not resemble the mottling caused by excessive fluorine in the diet but from preliminary observations resembles a deposit of pigment.

In table 3 the average tooth condition of the four groups after 26 weeks of supplement has been summarized. This

TABLE 3

*Condition of eyes and teeth of albino rats receiving different amounts of vitamin A*

GROUP	NO. ALIVE	TOOTH CONDITION AT 26 WEEKS	TOOTH GRADE AVE	EYE CONDITION AT 26 WEEKS
A	..	All abnormal <sup>1</sup>	..	96% abnormal <sup>1</sup>
B	15	All showed severe abnormalities	3.33	74% showed abnormalities
C	19	80% showed abnormalities	2.05	40% showed abnormalities
D	19	95% normal — very slight abnormality in 1 case	0.08	95% normal — 1 animal abnormal

<sup>1</sup> Condition at death — prior to 26 weeks.

time period was chosen since most of the animals in groups B, C, and D were alive at this time and since it was typical of the entire period for each group. It is apparent from the data that the severity of the tooth abnormalities is inversely related to the vitamin A intake. Of the levels studied normal teeth were produced only at the 20-unit dosage. It is entirely possible that a level between 4 and 20 units would have sufficed. Photographs of the various degrees of tooth abnormalities observed in this experiment are to be found in figure 2.

*Eyes.* In table 3 the average eye condition at 26 weeks or at death (whichever occurred first) has been recorded. The same general picture is again borne out — a decrease in abnormali-

ties with an increase in vitamin A intake. Normal eyes in 95% of the cases were obtained only at the 20-unit level. The one case of eye abnormality in this group was not necessarily due to A deficiency for animals in this colony on a high A intake occasionally exhibit a reddish exudate from the eye which persists over long periods.

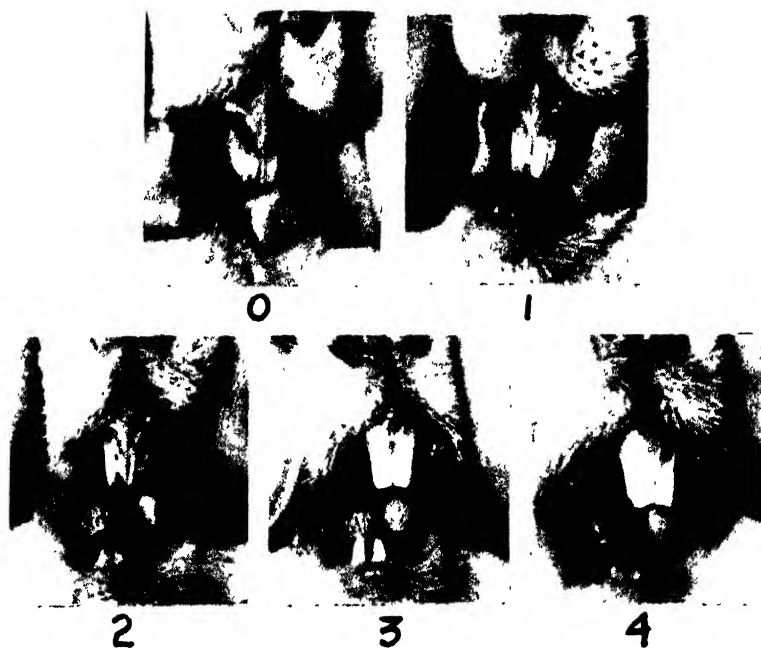


Fig. 2 Teeth showing various degrees of abnormality.

0 Normal, translucent, orange-pigmented tooth of rat 43♂—group D. 20 U.S.P. units of vitamin A per 100 gm. of body weight.

1 Opaque, light-yellow tooth of rat 39♀—group C—4 U.S.P. units of vitamin A per 100 gm. of body weight.

2 Light-yellow color tooth with slight black mottling of rat 13♂—group C—4 U.S.P. units of vitamin A per 100 gm. of body weight.

3 Chalky-white tooth of rat 50♀—group B—2 U.S.P. units per 100 gm. of body weight.

4 Chalky-white tooth with black mottling of rat 11♂—group B—2 U.S.P. units per 100 gm. of body weight.

## DISCUSSION

In these experiments an allowance of vitamin A for the albino rat of 2 U.S.P. units per 100 gm. of body weight allowed only suboptimal growth and cut the life expectancy to nearly one-third of that attainable on the same diet with increased vitamin A intake. The 2-unit level was also insufficient for normal tooth structure and healthy eyes.

From our data it may be estimated that the vitamin A requirement of the albino rat lies between 4 and 20 U.S.P. units daily per 100 gm. of body weight. Although no levels higher than 20 were studied, it is believed that this level equals or exceeds the requirement since (1) normal tooth and eye structure was produced at this level and (2) a five-fold increase in vitamin A above the 4-unit level produced approximately a 20% increase in body weight and a 25% increase in life span, whereas the 4-unit level compared with the 2-unit level (i.e., only a two-fold increase of vitamin A) produced an increase of 120% in life span and an increase of 20% in body weight. This has been graphically represented in figure 3 and from this graph the vitamin A requirement of the albino rat may be estimated at about 10 U.S.P. units per 100 gm. of body weight per day (i.e., the point approaching the flat portion of the curve). The requirement for normal teeth and eyes apparently lies in the same general range. The data indicate that optimal growth might be used as a criterion for the establishment of a vitamin A requirement adequate for all physiological functions.

This value of 10 U.S.P. units greatly exceeds the requirement of 1.8–2.2 U.S.P. units per 100 gm. body weight estimated from the data on vaginal smear records (Goss and Guilbert, '39). Better agreement is found with the values which just permit storage (7 to 17 U.S.P. units per rat found by Baumann, Riising and Steenbock, '34) or which permit optimum growth and very slight storage (25 U.S.P. units per rat as determined by Lewis et al., '42). Horton's ('41) work on the amount of vitamin A necessary to maintain blood levels also indicates that 20 I.U. of vitamin A represents the daily re-

quirement of the rat but does not allow storage. Horton reports 4 I.U. per rat daily as sufficient for the cure of xerophthalmia. In our experiment, 4 U.S.P. units per 100 gm. body weight gave relief of xerophthalmia in 60% of the animals.

It is believed that in establishing nutritional formulae a value for vitamin A should be used which is adequate to cover all physiological functions. Our experiments indicate that for

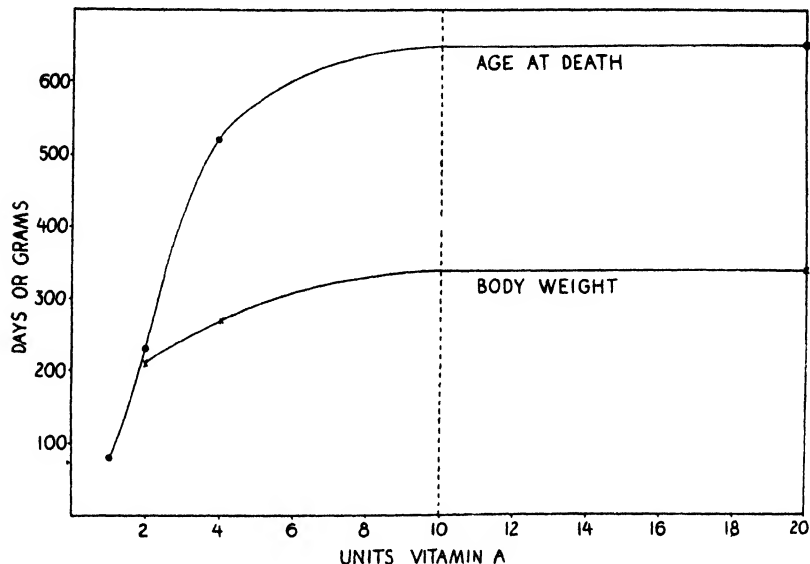


Fig. 3 Relationship between vitamin A intake and body weight, x———x body weight of rats at 26 weeks. Relationship between vitamin A intake and age at death, o———o average age at death of rats.

the albino rat this value is approximately 10 U.S.P. units of vitamin A per 100 gm. body weight. Calculation of the data of other workers to a body weight basis indicates fair agreement with this value.

#### SUMMARY

On a vitamin A-free diet the life span of albino rats increases with increasing vitamin A intake at levels of 1, 2, 4 and 20 U.S.P. vitamin A units per 100 gm. of body weight.

The effect of increasing vitamin A levels is reflected similarly on the four physiological criteria studied — life span, growth, tooth structure, and eye condition.

The vitamin A requirement of the albino rat estimated on the basis of maximum life span and optimal growth may be approximated at 10 U.S.P. units of vitamin A per 100 gm. of body weight. This is about five times the minimum requirement previously estimated on the basis of vaginal smear records.

#### ACKNOWLEDGMENT

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# NICOTINIC ACID DEFICIENCY IN TURKEY POULTS AND THE OCCURRENCE OF PEROSIS<sup>1</sup>

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## ONE FIGURE

(Received for publication August 3, 1945)

Briggs, Mills, Elvehjem, and Hart ('42) have shown that young chicks require a dietary source of nicotinic acid when fed highly purified diets. This finding has been extended to another avian species, the turkey, and the results, presented in this paper, show that the poult also requires nicotinic acid in the diet. Symptoms of a deficiency include poor growth, inflammation of the mouth, diarrhea, and perosis.

This observation extends the ever-growing list of animals which have been shown to require a dietary source of nicotinic acid since the discovery of the vitamin nature of this compound by Elvehjem, Madden, Strong, and Woolley ('38).

## EXPERIMENTAL

Day-old turkey poults (crossbred), obtained from the Poultry Department, were divided into uniform groups and reared

<sup>1</sup> Scientific paper no. 105. Contribution no. 1981 of the Maryland Agricultural Experiment Station (Department of Poultry Husbandry).

The author is indebted to Merck and Co., Inc., Rahway, N. J., for the crystalline vitamins; Wilson and Co., Inc., Chicago, Ill., for gelatin; The Wilson Laboratories, Chicago, Ill., for Liver Fraction I; Allied Mills, Inc., Portsmouth, Va., for soy-bean oil; Abbott Laboratories, North Chicago, Ill., for Haliver oil; E. I. du Pont de Nemours and Co., New Brunswick, N. J., for irradiated 7-dehydro-cholesterol; to Mr. Stanley Weaver and Mr. Carl Spitzer for assistance in various phases of the work, and to Dr. M. A. Jull for valuable suggestions.

in electrically heated battery cages with raised wire floors. Feed and water were given *ad libitum*. Weighings and other observations were made weekly and the experiments conducted for a 4-week period.

The basal ration, low in nicotinic acid but complete in all other nutrients, was composed as follows: glucose ("Cere-lose") 61.4, casein (Vitamin Test GBI) 18, gelatin 10, l-cystine 0.3, soybean oil 4, and Salts 1 M<sup>2</sup> 6. Each 100 gm. also contained the following amounts of vitamins: thiamine HCl 0.4 mg., riboflavin 0.8 mg., Ca pantothenate 2.0 mg., choline HCl 250 mg., pyridoxine HCl 0.6 mg., biotin 0.02 mg., i-inositol 100 mg., p-aminobenzoic acid 0.2 mg., 2-methyl-1, 4-naptho-quinone 0.1 mg., and alpha-tocopherol 0.5 mg. Vitamin A and vitamin D<sub>3</sub> (1200 U.S.P. units and 170 A.O.A.C. units, respectively) were fed by dropper weekly. Unidentified factors were supplied by a norite eluate concentrate of liver, containing vitamins B<sub>10</sub>, B<sub>11</sub>, and B<sub>12</sub>, prepared from Liver Fraction L (Wilson) by the method of Hutchings, Bohonos, and Peterson ('41). The amount of this fraction added was equivalent to 4% of original Liver Fraction L (or about 0.2% dry matter). The amount of nicotinic acid in the basal ration was not determined but since it is nearly identical with the ration used by Briggs et al. ('42) it probably contained not more than 0.3 mg. of nicotinic acid per 100 gm.

## RESULTS

A summary of four different experiments is given, for the sake of simplicity, in table 1. It can be seen that poult chicks receiving the basal ration grew poorly, with some deaths, and developed an inflammation of the mouth parts (similar to chick blacktongue). In addition, poult chicks receiving the basal

<sup>2</sup> Salts 1 M are composed of the following ingredients by weight:

CaCO <sub>3</sub>	150.0	MgSO <sub>4</sub> ·7H <sub>2</sub> O	50.0	ZnCl <sub>2</sub>	0.2
K <sub>2</sub> HPO <sub>4</sub>	90.0	Fe(C <sub>6</sub> H <sub>5</sub> O <sub>7</sub> ) <sub>2</sub> ·6H <sub>2</sub> O	14.0	CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.2
Na <sub>2</sub> HPO <sub>4</sub>	73.0	MnSO <sub>4</sub> ·4H <sub>2</sub> O	4.1	H <sub>2</sub> BO <sub>3</sub>	0.09
Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub>	130.0	KI	0.4	CoSO <sub>4</sub> ·7H <sub>2</sub> O	0.01
NaCl	88.0				600.0

When fed at a level of 6%, the mixture supplies 1.11 gm. of calcium, 0.58 gm. of phosphorus, and 0.01 gm. of manganese per 100 gm. of ration.

ration had poor feathers, diarrhea, low feed consumption, and low efficiency of feed utilization (.334<sup>3</sup> as compared with the control value of .626).

As the level of nicotinic acid was increased in the ration, growth likewise increased until a level of 3 to 5 mg. per 100 gm. was reached. There was a marked decrease in the incidence of inflammation of the mouth with the addition of 1 mg. of nicotinic acid per 100 gm. of ration and higher amounts prevented this trouble entirely. Thus, a greater level of nicotinic acid is required for growth than for the prevention

TABLE 1

*The effect of nicotine acid intake on growth, inflammation of the mouth, and perosis in turkey poult.*

GROUP	SUPPLEMENT TO BASAL RATION (NICOTINIC ACID LOW)	NO. STARTED	NO. ALIVE AT 4 WKS	AVERAGE WEIGHT AT 4 WKS. (WITH RANGE) GM	NO. WITH INFLAM- MATION OF MOUTH	NO. WITH PER- OSIS
1	None	19	13	114 (84-144)	14	6
2	1 mg. nicotinic acid/100 gm	6	6	154 (120-190)	1	3
3	2 mg. nicotinic acid/100 gm	13	11	267 (170-358)	0	9
4	3 mg. nicotinic acid/100 gm	13	13	332 (175-433)	0	4
5	4 mg. nicotinic acid/100 gm.	7	7	345 (300-430)	0	0
6	5 mg. nicotinic acid/100 gm.	20	20	350 (242-466)	0	1
7	10 mg. nicotinic acid/100 gm.	6	6	370 (287-405)	0	0

of mouth inflammation. The 10-mg. level of nicotinic acid gave slightly better growth than the 5-mg. level. This difference, however, is not thought to be significant. A larger number of poult would have to be used in order to establish the exact level of nicotinic acid required.

*Perosis.* It will be noted in table 1 that the poult receiving low levels of nicotinic acid had perosis (fig. 1). The incidence of perosis was highest in the groups receiving 2 mg. of nicotinic acid and was also more severe in these groups, often involving an actual slipping of the tendon of Achilles from its

<sup>3</sup> Total weight gained

Total feed consumed



condyle. Higher levels of nicotinic acid caused a marked reduction of incidence of perosis. It is evident, therefore, that nicotinic acid is required by the poult for the prevention of perosis in addition to manganese (Ringrose, Martin and Insko, '39), choline (Jukes, '40), and biotin (Patrick, Boucher, Dutcher and Knandel, '43).



Fig. 1 A typical case of perosis in the turkey poult due to too low a level of nicotinic acid in the diet.

#### DISCUSSION

The amount of nicotinic acid required by the turkey poult (at least 3 to 5 mg. of nicotinic acid per 100 gm. of ration) is not less than two or three times higher than the minimum level required by the White Leghorn chick (Briggs et al., '42). This is not especially surprising since it has been found that the turkey poult's requirements for most of the other vita-

mins is higher than that for the chick. It should be emphasized that the nicotinic acid deficiency in the poult was produced by the use of a highly purified diet and that the results do not show whether or not the requirement for nicotinic acid by poult receiving practical rations is the same.

The perosis obtained in these experiments might be expected from studies with young chicks which have shown that a nicotinic acid deficiency in a ration, adequate in all other respects, may result in perosis (Briggs, Luckey, Tepley, Elvehjem and Hart, '43, and unpublished data). The occurrence of perosis due to this deficiency may explain the perosis obtained in poult by Patrick et al. ('43) which they attribute to an unrecognized factor in yeast "which can be adsorbed on fuller's earth or norite and subsequently eluted with ammonium hydroxide." The simplified rations which they used appear to be low in nicotinic acid. Evans, Rhian and Draper ('43) have reported that turkey poult, receiving a natural type of diet, require another factor or factors for the prevention of perosis other than manganese and choline.

It is recognized that still other factors may be required for the prevention of perosis. If still another unidentified factor (or factors) is required, it must be contained in the norite eluate concentrate of the basal ration (or as an impurity in the other purified ingredients).

#### SUMMARY

Turkey poult, when fed a highly purified diet containing all necessary nutrients except nicotinic acid, grew poorly and developed certain deficiency symptoms such as inflammation of the mouth, diarrhea, low feed consumption, poor efficiency of feed utilization, poor feathering, and perosis. The perosis in the deficient birds occurred in spite of ample manganese, choline, and biotin in the diet. All symptoms were prevented by the addition of 3 to 5 mg. of nicotinic acid per 100 gm. of ration. Higher levels of nicotinic acid, however, may be needed for optimum growth.

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# FACTORS AFFECTING THE DIETARY NIACIN AND TRYPTOPHANÉ REQUIREMENT OF THE GROWING RAT <sup>1</sup>

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ONE FIGURE

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That the dietary requirement of a single nutrient can be rather variable and dependent upon the character and quality of the accompanying nutrients is becoming increasingly evident. Such interplay of nutrients is classically exemplified by the sparing action of fat on thiamine (Evans, Lepkovsky and Murphy, '34) and the increased dietary riboflavin requirement for the rat on high fat rations (Mannering, Lipton and Elvehjem, '41). The effect of the type of carbohydrate on intestinal synthesis of undifferentiated B complex factors is evident from the coprophagy studies of Guerrant, Dutcher and Tomey ('35) and Guerrant, Dutcher and Brown ('37). The effect of these extrinsic variables which so markedly alter the physiology of the organism, has resulted in an increased interest in the importance of these factors to the host. Krehl, Teply and Elvehjem ('45) have shown that when corn is added

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to synthetic rations, the nicotinic acid requirement of the dog is increased. In addition it has been demonstrated (Krehl, Teply and Elvehjem, '45) that corn is an etiological factor in the production of a nicotinic acid deficiency in the rat, a species which heretofore had not been shown to require a dietary source of this vitamin. This deficiency was further complicated by the fact that increased levels of casein eliminated the deleterious effect and that tryptophane alone was effective on low casein diets (Krehl, Teply, Sarma and Elvehjem, '45). From these results it is evident that an interrelationship between a vitamin (nicotinic acid) and an amino acid (tryptophane) exists. Although this phenomenon appears to be new in animal nutrition it is not so in bacterial nutrition as Stokes and Gunness ('45) have shown that pyridoxine and pyridoxamine and certain amino acids have a dual role for some *Lactobacilli*.

This investigation was carried out to obtain further information on the interchangeable role of nicotinic acid and tryptophane and the relationship of other ration components to the dietary tryptophane requirement.

#### EXPERIMENTAL AND RESULTS

##### *General*

The composition of the nicotinic acid low basal rations used is given in table 1. Vitamins were incorporated in all these rations, unless otherwise indicated, at the following levels: thiamine chloride 0.2, riboflavin 0.3, pyridoxine HCl 0.25, calcium pantothenate 2.0, choline chloride 100, inositol 10, 2-methyl-naphthaquinone, 0.1 and biotin 0.01 mg. per 100 gm., respectively. Halibut liver oil diluted 1:2 with corn oil was fed at a level of 2 drops per week, with  $\alpha$ -tocopherol included at 0.5 mg. per drop. A norite eluate of solubilized liver extract, prepared so as to contain practically no nicotinic acid was fed where indicated. This preparation was made by adjusting the norite eluate to pH 3, followed by the addition (in the cold) of absolute ethanol to make a concentration of 90% by

TABLE 1  
*Composition of basal rations used.*

RATION CONSTITUENT	RATION NO.																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Casein, gm. <sup>1</sup>	15	20	15	15	15	15	15											
Gelatin, gm.										10	10	10	10	10	10	9	9	9
Wheat gluten, gm. <sup>2</sup>										15	15	15	15	20				
Corn starch, gm.												68	68			84		
Dextrin, gm.			78							68						84		
Glucose, gm.				78														
Lactose, gm.					26													
Sucrose, gm.	78	73			52	55	55	42	52	68			68					84
Corn oil, gm.	3	3	3	3	3		25		6	3	3	3	3	3	2	3	3	3
Butter fat, gm. <sup>3</sup>						25												
Salts IV, gm. <sup>4</sup>	4	4	4	4	4	5	5	2	2	4	4	4	4	4	3	4	4	4
1 (—) Cystine, gm.	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15						0.2	0.2	0.2
Klim, gm. <sup>5</sup>								56										
Skin milk powder									40									
Refined wheat flour <sup>6</sup>																		85

Generous levels of vitamins (see text) were supplied in all rations.

<sup>1</sup> Extracted 3 X with hot 95% ethanol.

<sup>2</sup> Fed at a level to allow 15% protein.

<sup>3</sup> Washed twice with hot water.

<sup>4</sup> Phillips and Hart ('35).

<sup>5</sup> A commercial whole milk powder.

<sup>6</sup> Unenriched refined wheat flour.

volume. The voluminous precipitate was filtered off, redissolved in the least amount of water necessary, the pH readjusted to 3, and the precipitation repeated as above. By this means nearly all of the nicotinic acid was removed with a minimum loss of "folic acid" activity. The corn products

TABLE 2

*Protein, tryptophane and nicotinic acid contents of dietary supplements.*

SUPPLEMENT	PROTEIN, TRYPTOPHANE AND NICOTINIC ACID CONTENT OF SUPPLEMENT		
	Protein (N× 6.25)	Tryptophane	Nicotinic acid
	%	mg. %	mg. %
Corn grits	9.0	25	0.65
Yellow corn	8.5	52	2.28
White corn	9.2	55	2.25
Ethanol extracted yellow corn	7.2	43	1.30
Polished rice	7.5	75	1.40
Soybean flour	50.0	600	2.00
Whole wheat	15.0	150	5.50
Refined wheat flour <sup>1</sup>	13.5	108	1.0
Whole rye flour	12.5	125	1.05
Rolled oats	12.5	150	1.05
Skim milk powder	37.5	450	1.05
Klim <sup>2</sup>	26.8	322	0.66
Whole milk	3.4	54	0.08
Casein	100.	1200	< 0.01
Wheat gluten	88.	800 <sup>3</sup>	1.62
Gelatin	105.	0.0	< 0.01
Corn starch	.	.	.

<sup>1</sup> Unenriched white flour.

<sup>2</sup> A commercial whole milk powder.

<sup>3</sup> Calculated as 0.8%, based on 100% protein, calculated to 16% nitrogen.

and other cereals (table 2) were added to the extent of 40% of the entire basal ration. The vitamin and cystine levels were maintained as previously indicated in both the basal and supplemented rations. The counteracting effect of nicotinic acid and other substances was tested by incorporating them in the supplemented ration unless otherwise specified.

All the crude materials were ground to a consistency comparable to the rest of the ration and analyzed for crude protein ( $N \times 6.25$ ) and nicotinic acid (table 2). The protein and nicotinic acid content of the various rations presented in table 3 are calculated on the basis of analytical results given in tables 2 and 3. The tryptophane values given in tables 2 and 3 are based on the best and latest available analytical data given by Block and Bolling ('43, '45). All rations were fed ad libitum to weanling rats of the Sprague Dawley strain. The growth period employed was 5 weeks unless otherwise indicated, and the growth results are presented in grams gained per week with the range in parentheses. The growth data obtained in the various experiments are presented in table 3.

Although the results are given only for the sucrose basal, growth was very good (28–30 gm. per week for 5 weeks) irrespective of the basal ration used (table 1) with the exception of rations 10 through 18 (to be discussed later). A growth performance markedly below that obtained on any of the basal rations then must be considered due to the deleterious action of the supplement used. To generalize further the addition of 1 to 1.5 mg.% of nicotinic acid (or 50 mg.% of tryptophane) to a corn supplemented ration invariably resulted in growth equal or superior to that obtained on the basal ration.

The results obtained with the basal rations, which contain practically no nicotinic acid clearly demonstrate that a dietary source of this vitamin is not required by the rat. Since the basal rations cannot be formulated to the same composition as the supplemented rations, results obtained with them are not to be interpreted as direct controls, but rather as valuable indices in interpreting growth results. Comparison of the growth performance on the corn-supplemented rations and these rations plus nicotinic acid or other corrective supplements, is however, a direct one.



TABLE 3  
Growth results on various diets.

GROUP NO.	BASAL RATION		NICO-TINIO ACID ADDED	TRYPTO-PHANE ADDED	TOTAL PROTEIN	TOTAL TRYPTO-PHANE	TOTAL NICO-TINIO ACID	GAIN PER WEEK <sup>4</sup> (RANGE)
(TABLE 1) + ADDED SUPPLEMENT (TABLE 2)								
			+ or -	+ or -	%	mg. %	mg. %	gm.
1	1		-	-	15.0	180	< 0.01	28 (25-32)
2	1	Corn grits	-	-	12.6	118	0.27	7 (4-11)
3	1	Corn grits	+	-	12.6	118	1.27	29 (24-35)
4	1	Yellow corn	-	-	12.4	129	0.92	13 (11-16)
5	1	White corn	-	-	12.7	130	0.91	14 (7-16)
6	1	Polished rice	-	-	12.0	138	0.56	31 (27-34)
7	1	Soybean flour	-	-	29.0	348	0.80	26 (21-38)
8	1	Whole wheat	-	-	15.0	168	2.20	31 (31-31)
9	1	Refined wheat flour	-	-	14.4	151	0.40	29 (26-30)
10	1	Rye flour	-	-	14.0	158	0.41	26 (24-27)
11	1	Rolled oats	-	-	14.0	168	0.41	28 (26-29)
12	4	Corn grits	-	-	12.6	118	0.27	23 (18-29)
13	3	Corn grits	-	-	12.6	118	0.27	21 (13-26)
14	5	Corn grits	-	-	12.6	118	0.27	15 (12-17)
15	1	Corn starch	-	-	9.0	108	< 0.01	19 (16-22)
16	1	Corn grits + norite eluate <sup>1</sup>	-	-	12.6	118	0.27	6 (5-8)
17	1	(low vitamins) <sup>2</sup>	-	-	15.0	180	< 0.01	21 (17-27)
18	1	Corn grits (low vitamins) <sup>2</sup>	-	-	12.6	118	0.27	7 (5-10)
19	1	Corn grits (low vitamins) <sup>2</sup>	+	-	12.6	118	1.27	20 (18-21)
20	4	(low vitamins) <sup>2</sup>	-	-	15.0	129	< 0.01	28 (26-31)
21	4	Corn grits (low vitamins) <sup>2</sup>	-	-	12.6	118	0.27	15 (11-18)
22	4	Corn grits (low vitamins) <sup>2</sup>	+	-	12.6	118	1.27	24 (21-29)
23	1	Corn grits (high vitamins) <sup>3</sup>	-	-	12.6	118	< 0.01	6 (6-7)
24	1	Corn grits (high vitamins) <sup>3</sup>	+	-	12.6	118	1.77	20 (19-22)
25	1	Corn grits + 5 cc. milk/day	-	-	12.7	121	0.28	18 (9-26)
26	1	Corn grits + 10 cc. milk/day	-	-	12.9	124	0.28	23 (21-24)
27	9	Yellow corn	-	-	12.4	129	1.16	31 (26-34)
28	8	Corn grits	-	-	12.6	118	0.48	28 (25-31)
29	6	Corn grits	-	-	12.6	118	0.27	10 (9-12)
30	7	Corn grits	-	-	12.6	118	0.27	12 (10-14)
31	2	Yellow corn	-	-	15.4	165	0.92	30 (22-35)
32	1	Corn grits	-	+	12.6	168	0.27	31 (27-37)
33	1	Corn grits	-	+	12.6	218	0.27	33 (26-35)
34	1	Corn grits	+	-	12.6	118	1.77	33 (31-36)
35	10		-	-	25.0	120	0.25	7 (4-12) <sup>4</sup>
36	10		+	-	25.0	120	1.25	23 (21-28)
37	10		-	+	25.0	145	0.25	22 (17-26)
38	14		-	-	30.0	160	0.32	26 (24-29)
39	11		-	-	25.0	120	0.25	21 (17-24)
40	12		-	-	25.0	120	0.25	18 (18-18)
41	13		-	-	25.0	120	0.25	13 (8-18)
42	13		+	-	25.0	120	1.25	23 (19-26)
43	15		-	-	21.5	92	0.85	23 (18-26)
44	15		+	-	21.5	92	1.85	30 (29-31)
45	16		-	-	9	108	< 0.01	24 (21-28)
46	17		-	-	9	108	< 0.01	26 (24-28)
47	18		-	-	9	108	< 0.01	14 (12-15)
48	18		+	-	9	108	1.5	17 (17-18)
49	18		-	+	9	158	< 0.01	21 (19-23)

<sup>1</sup> Norite eluate = 23  $\mu$ g. B. (*S. lactis* assay) per 100 gm. of ration.

<sup>2</sup> (low vitamins) — general vitamin level reduced to 60% of that given in text.

<sup>3</sup> (high vitamins) — general vitamin level increased to 3 times that given in text.

<sup>4</sup> Three animals used per group. Most experiments were repeated.

<sup>5</sup> 4-weeks growth period from weaning. This applies to all groups 35-49, inclusive — the rest of the items given in this column.

*Effect of varieties of corn and other cereals on the growth of rats*

Since corn grits are consumed in large quantities in the southern part of the United States and since they produce the most serious growth retardation (table 3) most of the work reported was done with this product. It can be seen (table 3, groups 4 and 5) that yellow or white corn do not retard growth as severely as corn grits. This may be due to the combined effect of the larger nicotinic acid and tryptophane content of these materials. Nicotinic acid in every case counteracted these growth depressions.

Corn grits which were heated (dry) in the autoclave for 1 hour at 15-pounds pressure still produced very poor growth. Ground white corn was reduced to the consistency of ordinary white flour by grinding for 24 hours in a ball mill, yet no significant improvement in growth could be observed as a result of this treatment.

In addition to corn, other cereals, namely polished rice, whole wheat flour, refined wheat flour, whole rye flour, whole rolled oats, and soybean flour were tested in a comparable manner. Good growth resulted in every case (table 3, groups 6 through 11). It is interesting to note that though polished rice, rolled oats, and rye, all contain significantly less nicotinic acid than yellow corn, yet they did not produce growth depression. This fact may in part be attributed to a higher tryptophane content.

*The role of carbohydrate and vitamin level in counteracting the growth depressing action of corn*

Inasmuch as it has been shown (Mannering, Orsini and Elvehjem, '44; Schweigert, McIntire, Henderson and Elvehjem, '45) that the type of carbohydrate used in a synthetic ration has a marked influence on vitamin synthesis in the intestinal tract, the effect of corn was tested when fed with sucrose, glucose, dextrin and lactose. When lactose was used the carbohydrate portion of the ration contained two-thirds

sucrose and one-third lactose, since rats fail to grow on a high lactose low-fat ration (Schantz, Elvehjem and Hart, '38). Starch was also used, but in this case it was added to the basal ration in the usual manner to the extent of 40%. The growth results obtained using various carbohydrates are shown in table 3, groups 12, 13 and 14, and it is evident that glucose, dextrin and to a lesser degree, lactose exert a marked influence in modifying the undesirable action of corn grits. These observations strongly indicate that corn produces alterations in the intestinal flora and that the type of carbohydrate used may be important in determining the extent of these changes. This theory is given additional support by the fact that when corn starch alone is added to the basal ration to the extent of 40% reasonably good growth is obtained (table 3, group 15), even though the nicotinic acid content is negligible and the 9% casein in this ration supplies several of the essential amino acids below the minimum indicated by Rose ('37). Furthermore, animals which received glucose, dextrin or lactose show a pronounced variability in growth not only between animals in the same group but at different points in the growth period, with poor growth occurring at first, followed by rapidly increasing growth from week to week. This variation might be due to a gradual favorable modification in the intestinal flora under the influence of the proper type of carbohydrate. Since Boutwell, Geyer, Elvehjem and Hart ('45) have shown that the level of vitamins is important in determining the growth of rats on high-fat rations, the vitamin level of the basal and corn-supplemented ration was lowered to 60% of the usual level. The results of this modification are noted in table 3, groups 17 through 22. When the vitamin level was lowered, glucose, which had previously exerted a protective action against corn, now failed to allow adequate growth on the corn-supplemented ration, although growth on the basal was normal. On the lowered vitamin level, growth on the sucrose basal was reduced from 28 to 21 gm. per week. Nicotinic acid was correspondingly

less efficient in counteracting the action of corn particularly when sucrose was used as the carbohydrate.

Since it is apparent that a lowered level of vitamins produces a significant effect, the vitamin level was tripled. It can be seen (table 3, groups 23 and 24) that no benefits were obtained from raising the general level of vitamins; in fact, the usual effectiveness of nicotinic acid was somewhat impaired. Norite eluate  $\cong$  to 23  $\mu$ g. of vitamin B<sub>6</sub> per 100 gm. of ration did not counteract the growth depression caused by corn; neither did p-aminobenzoic acid at a level of 25 mg. per 100 gm. of ration. Hence nicotinic acid alone of all the known members of the B complex factors seems to have a specific counteraction against the growth retarding effect of corn.

*Milk and milk products as corrective agents  
against the effect of corn rations*

Since Goldberger, Waring and Willets ('15) demonstrated that pellagra was a deficiency disease and advocated a good diet including adequate amounts of milk as a preventive (Goldberger and Tanner, '24), the value of milk in counteracting the deficiency caused by corn was ascertained.

When milk was fed at a level of 5 and 10 ml. per day as a supplement to the ration which contained corn, a marked beneficial effect was observed (table 3, groups 25 and 26). The addition of 5 and 10 gm. levels of whole milk powder per 100 gm. of ration also gave favorable results. In all cases where these small amounts of milk were used, growth was variable and quite often delayed, a phenomenon which also was seen on those carbohydrate rations which proved beneficial. In addition, the effect of these relatively small additions of milk could in every case be further improved by the addition of nicotinic acid. It should be emphasized, however, that the positive results that were obtained could not be related to the nicotinic acid or tryptophane content of the milk, but probably to an altered intestinal flora resulting from the feeding of milk. This difference in flora is obvious from a gross

observation of the enlarged cecum and of its contents. A skim milk powder ration (table 1, ration 9) and a whole milk powder ration (table 1, ration 8) were supplemented with yellow corn and corn grits, respectively. Excellent growth was obtained in both cases (table 3, groups 27 and 28). Again the results cannot entirely be attributed to the nicotinic acid or tryptophane content of these rations. These favorable results are probably due to a combination of several factors, including the high level of lactose.

Since Boutwell et al. ('45) have shown a nutritional difference between butter fat and corn oil, an experiment was designed to test the difference between these two fats in corn-supplemented rations. The high-fat basal rations (table 1, rations 6 and 7) which contain butter fat and corn oil, respectively, were supplemented with corn grits. Good growth was obtained with either basal ration, but neither butter fat or corn oil proved effective in counteracting the action of corn (table 3, groups 29 and 30).

*The efficacy of tryptophane in counteracting  
the action of corn*

That tryptophane is intimately concerned in the deficiency syndrome caused by corn has been demonstrated (Krehl et al., '45). Increased tryptophane ingestion, whether by increased protein level, or added l (—) or dl-tryptophane in amounts as low as 50 mg. per 100 gm. of ration is as effective as 1 or 1.5 mg. of nicotinic acid, in counteracting the deleterious effect of corn (table 3, groups 32, 33 and 34).

If nicotinic acid produces the observed effect by establishing a type of intestinal flora which favors the synthesis of protein (tryptophane) then small injections of nicotinic acid should have a limited action providing excretion into the intestine does not occur. If in the same manner, a tryptophane deficiency is the critical factor in this syndrome, the injection of this substance in amounts comparable to those effective in the

ration, should prove completely adequate in correcting the deficiency.

To test this idea four groups of rats (3 rats per group) that had received the corn supplemented ration were treated as follows: Group 1 received 0.1 mg. of nicotinic acid daily (this amount received daily in the diet is adequate), group 2 received 0.2 mg. of nicotinic acid daily while group 3 was given 5 mg. of tryptophane daily. The tryptophane and nicotinic acid supplements were injected each day subcutaneously. The fourth group was maintained untreated as a control. After 1 week the growth results obtained in grams gained per day were as follows: Group 1 receiving 0.1 mg. nicotinic acid, 2.4; group 2 getting 0.2 mg. nicotinic acid, 4.3; group 3 with 5 mg. tryptophane, 6.6; and group 4, the control, 0.55.

It is evident that the performance of animals receiving tryptophane injections is superior particularly when compared to the lower level of nicotinic acid. This line of evidence, while favoring the idea that a tryptophane deficiency is paramount in this problem, is not conclusive. Again we cannot be sure to what extent excretion of the injected nicotinic acid or tryptophane into the intestinal tract modifies the above results.

*Counteraction of corn by various levels of nicotinic acid  
and nicotinic acid derivatives*

Numerous levels of nicotinic acid were tested to determine the range of effectiveness of this vitamin in counteracting the deficiency caused by feeding corn. Levels ranging from 0.05 to 1.5 mg.% were tested and the results plotted in figure 1. It is apparent that the first level of nicotinic acid to show a significant effect is 0.4 mg.%. This level is based on sucrose as the carbohydrate and might be different when other carbohydrates are employed.

The following derivatives of nicotinic acid were also tested: nicotinamide, ethyl nicotinate, N-phenyl nicotinamide, trigonelline and nicotinamidemethochloride. These derivatives

were fed on a molar equivalent basis equal to 1.5 mg.% of nicotinic acid. Of these compounds, only nicotinamide and ethyl nicotinate were active. These results correlate with the action of these substances in curing blacktongue in the dog (Woolley, Strong, Madden and Elvehjem '38). N-phenyl nicotinamide appears to be inactive for the rat whereas it is

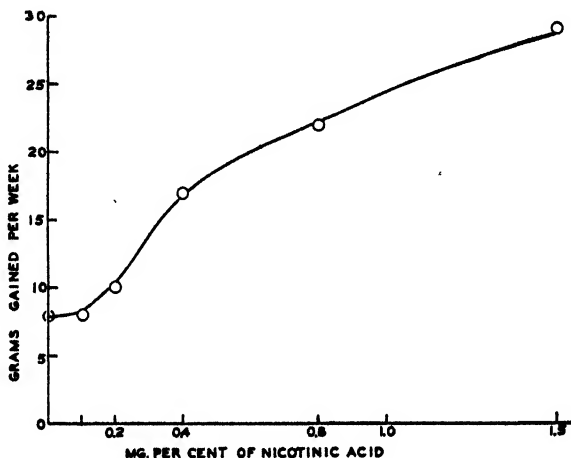


Fig. 1 The effect of various levels of nicotinic acid in counteracting the growth depression caused by corn.

about 25% as active as nicotinic acid for the dog. It is interesting to note that nicotinamidemethochloride is as inactive for the rat as for the dog (Teply, Krehl and Elvehjem, '45). This is in contradiction to the observation of Najjar, Hammond, English, Worden and Deal ('44), concerning the compound.

*Additional observations on the deleterious  
effect of corn*

Since all of the above experiments were done with male rats, the same experimental conditions were applied to a series of female rats in which case growth depression from corn and counteraction by nicotinic acid took place in a similar manner.

In addition to the growth depression observed in the corn-fed young growing rat, the animals appeared unkempt, with a rough fur coat and with occasional alopecia. Also the reddish accumulation of porphyrin like material on the paws, nose and whiskers of deficient rats described by Chick, Macrae and Worden ('40) was observed in all but a small percentage of the above animals.

To test the effect of corn supplemented rations on matured rats a group of adult males which weighed 250–285 gm. were given this ration for a period of 10 weeks. At the end of this time the animals weighed 330–407 gm. and showed no symptoms of any kind comparable to those seen in the young growing rat.

In an attempt to correlate an altered intestinal flora with differences in the amounts of nicotinic acid, "folic acid" and tryptophane found in the cecal contents of rats on the various diets, animals were sacrificed by decapitation, the cecal contents removed and autoclaved for 1 hour at 15-pounds pressure in 0.1 N KOH. The livers were removed at the same time, treated in a similar manner and analyzed for nicotinic acid. "Folic acid" was determined by the method of Teply and Elvehjem ('45), nicotinic acid by the method of Krehl, Strong and Elvehjem ('43) and tryptophane microbiologically by the omission of tryptophane and the inclusion of nicotinic acid in the medium of Krehl et al. ('43). The results of these analyses (table 4) clearly demonstrate that appreciable amounts of nicotinic acid are present in the cecal contents and that these amounts increase in those cases in which the carbohydrate used proved beneficial. The amounts of tryptophane and "folic acid" present in the cecal contents appear not to be markedly affected by the diet. It is rather difficult to deduce from this technique how much of the respective nutrient has been or is being made available to the host. The low nicotinic acid content of the livers of rats which received corn grits correlates well with retarded growth, and in every case in which nicotinic acid was added or good growth resulted from



TABLE 4

*Analysis of cecal contents and liver.*

BASAL RATION (TABLE 1)	+ SUPPLEMENT (TABLE 2)	NICOTINIC ACID ADDED	GRAMS GAINED PER WEEK <sup>1</sup>	ANALYSES OF CECAL CONTENTS <sup>1</sup>				LIVER ANALYSIS			
				Nicotinic acid		Tryptophane		"Folic acid"		Nicotinic acid	
				$\mu\text{g./gm.}$	Total	$\mu\text{g./gm.}$	Total	$\mu\text{g./gm.}$	Total	$\mu\text{g./gm.}$	Total
1		+ or -		$\mu\text{g.}$	$\mu\text{g.}$	$\mu\text{g.}$	$\mu\text{g.}$	$\mu\text{g.}$	$\mu\text{g.}$	$\mu\text{g.}$	$\mu\text{g.}$
1	Corn grits	-	26	24	38	278	450	1.01	1.60	161	1626
1		-	6	30	44	269	387	2.27	3.09	106	420
1	Corn grits	+	26	39	67	245	430	2.17	3.60	159	1523
1	Corn grits <sup>2</sup>	-	7	30	38	317	397	1.45	2.23	109	398
1	Corn grits <sup>2</sup>	+	24	38	83	259	563	2.11	4.50	160	1345
1	Corn grits <sup>2</sup>	-	14	33	56	270	473	1.94	3.90	141	791
1	Corn grits <sup>4</sup>	-	19	34	66	265	515	1.95	3.45	135	1094
4	Corn grits	-	20	35	42						
4	Polished rice	-	32	46	79						
4	Rollod oats	-	36	53	126						
8		-	31	37	207						
8	Corn grits	-	28	36	134						
3	Corn grits	-	22	37	120						
5	Corn grits	-	15	37	163						

<sup>1</sup> Average of group of 3 rats.<sup>2</sup> Norite eluate  $\approx$  11.5  $\mu\text{g. B.}$  per 100 gm. of ration.<sup>3</sup> Plus 2 ml. fresh milk per day.<sup>4</sup> Plus 5 ml. fresh milk per day.

feeding milk, the nicotinic acid content of the liver was elevated and was in the normal range. Whether the low values obtained are low enough to be a causative factor in the deficiency symptoms remains to be proven.

*The influence of the type of carbohydrate and nicotinic acid on the dietary tryptophane requirement*

Since it is evident that corn owes its deleterious action at least in part to the fact that it is deficient in tryptophane an effort was made to discover whether this action could be demonstrated with non-corn containing rations that were low in tryptophane. With this in mind rations 10 through 14 (table 1) were devised. Wheat gluten was the principle protein employed since it is low in tryptophane and gelatin was added to help compensate for other amino acid deficiencies, especially lysine. It is apparent from table 3, group 35 that rats fail to grow on this ration with sucrose as the carbohydrate unless nicotinic acid (group 36) or tryptophane (group 37) is added. Thus we find that corn is not unique in producing severe growth failure, and that the corrective interplay between nicotinic acid and tryptophane is not restricted to corn supplemented rations. Furthermore, we find a marked correlation in the protective action of dextrin and glucose in these rations, as in the corn rations, against the deleterious effect of a combined deficiency of tryptophane and nicotinic acid (table 3, groups 39 and 40). When corn starch was used as the carbohydrate in the wheat-gluten-gelatin ration (table 3, group 41) good growth was not obtained until nicotinic acid was added (group 42). It appears therefore that the efficacy of carbohydrate in counteracting the tryptophane deficiency decreases in the following order: dextrin, glucose, corn starch and sucrose. The addition of either nicotinic acid or tryptophane to rations which contain dextrin or glucose did not improve growth significantly which is indicative of their role in promoting an intestinal flora which adequately supplements a sub-optimum amount of dietary tryptophane. In this same

way, ration 15, (table 1) which contains 85% refined wheat flour resulted in good growth (table 3, group 43) despite its deficiency in tryptophane. The fair amount of nicotinic acid in this case is probably helpful which seems to be substantiated by the fact that additional nicotinic acid resulted in improved growth (group 44).

To further test the capacity of carbohydrate to modify the dietary tryptophane requirement, rations 16, 17 and 18 were prepared. Although the deficiency of tryptophane is paramount in these rations, other amino acids, particularly lysine, are also deficient. Despite this fact relatively good growth was obtained when dextrin or corn starch was employed as the carbohydrate (table 3, groups 45 and 46). Moreover, the addition of nicotinic acid or tryptophane did not benefit growth significantly. However, when sucrose was used as the carbohydrate, growth was poor and in this case the addition of nicotinic acid gave only partial improvement while tryptophane supported still better growth (table 3, groups 47, 48 and 49). This may be due to the fact that under these conditions sucrose is digested and absorbed so rapidly (Rabinowitch, '45) that it does not get into that part of the intestinal tract in which synthesis is most active.

Inasmuch as Albanese, Holt, Kajdi and Frankstone ('43) have shown that tryptophane plays an important role in maintaining an adequate level of total plasma protein and hemoglobin, analyses were made for these constituents. In addition, free plasma protein was determined. These results are compiled in table 5 and it is apparent that any direct correlation between growth failure and reduced plasma protein is made difficult due to the small differences obtained. Small differences are also evident in the hemoglobin values, while a consistent difference in the free plasma tryptophane is apparent between the animals which received corn and corn plus nicotinic acid, although both levels are in the normal range. At present it is impossible to explain the rather low plasma tryptophane values obtained on the wheat gluten

TABLE 5  
*Plasma protein, tryptophane and hemoglobin analysis.*

BASAL RATION (TABLE 1)	+ SUPPLEMENT (TABLE 2)	NIOTINIC ACID ADDED	TRYPTOPHANE ADDED	AVERAGE GRAMS GAINED PER WEEK <sup>1</sup>	AGE FROM WEANING	PLASMA PROTEIN	HEMOGLOBIN	TRYPTOPHANE μG./ML. PLASMA
		+ or -	+ or -		weeks	%	gm. %	
1		-	-	27	4	5.5	9.9	
1	Corn grits	-	-	9	4	3.2	7.6	
1	Corn grits	+	-	27	4	5.3	10.5	
1		-	-	28	6	5.5	11.0	
1	Corn grits	-	-	8	6	4.7	13.0	
1	Corn grits	+	-	30	6	4.8	12.2	
1	Corn grits	-	+	31	6	5.3	12.6	
1		-	-	30	7	5.3	12.8	
1	Corn grits	-	-	9	7	4.4	10.7	
1	Corn grits	+	-	32	7	5.5	12.9	
1	Corn grits	-	-	8	7			
1	Corn grits	+	-	30	7			
10		-	+.025%	22	4			14.7; 12.0
10		+	+.025%	23	4			22.0; 18.2
10		-	-	7	4			18.0
10		+	-	23	4			17.0
11		+	-	21	4			13.3
11		+	-	24	4			16.7
13		+	-	13	4			8
13		+	+	23	4			5.5
								7.0

<sup>1</sup> Average of group of 3 rats.

rations 11 and 13 which contained dextrin and corn starch, respectively.

#### DISCUSSION

While no complete explanation of the syndrome caused by feeding corn can be offered until the mechanism of the apparent interplay between nicotinic acid and tryptophane is elucidated, one can turn to the theory of an altered intestinal flora to explain most of the experimental observations. The effect of different carbohydrates on the growth depression might be explained on the grounds that they contribute to the establishment of an intestinal flora which is capable of synthesizing adequate amounts of the deficient factor. Of course, this does not explain why on nicotinic acid low synthetic rations, there is beneficial synthesis and normal growth, while changes in the intestinal flora and poor growth result from feeding corn. Again one might postulate a substance or substances in corn which combines with nicotinic acid or in some way makes it unavailable. Then too, Koser and Baird ('44) have demonstrated that a number of bacteria destroy nicotinic acid, and corn may well promote the development of such microorganisms. The fact that glucose which normally has a protective action against corn, gives much poorer protection when the vitamin level is lowered, again indicates an alteration of intestinal flora. This finding correlates with similar observations by Boutwell et al. ('45). Since small amounts of milk prove beneficial in counteracting the action of corn, one can reasonably assume a corrective action through re-establishment of a more favorable intestinal flora. The fact that the cecal contents are bulky, loose and quite liquid when milk is fed is probably due to the lactose.

Among the cereals tested corn seems to be quite specific in retarding growth of rats although rice, oats and rye which allow excellent growth are relatively poorer in nicotinic acid. The factor of a more adequate tryptophane supply probably accounts for this. It is also of interest to note that rats on the

rice-supplemented ration which contains only about 12% protein, grew 5 gm. per week more than did the animals on the soybean supplemented ration which contained 29% protein.

Although corn, like other cereals, contains starch, and although starch is a carbohydrate which seems to promote an intestinal flora favorable to the host, the supplementation of corn results in poor growth. The factors of low tryptophane and nicotinic acid concomitant with an unfavorable protein-carbohydrate relationship both as regards quality and quantity may be the cause of poor growth.

Despite the fact that both tryptophane and nicotinic acid are completely and separately active, the mechanism of this interplay is not understood. Upon analysis of the results in table 3 it is apparent that when corn is employed in the ration the dietary tryptophane is reduced to a level which does not provide growth. This situation can be corrected, however, by any of several means, namely by increasing the level of tryptophane to approximately 150 mg.% or the nicotinic acid to about 1.25 mg.%, or by using a type of carbohydrate (dextrin or glucose for example) which is known to promote intestinal synthesis. That these factors are clearly related and not a phenomenon peculiar to corn supplemented rations alone is evident from the fact that the same relationships hold true for the non-corn containing sucrose-wheat gluten-gelatin ration. A concomitant lysine or other amino acid deficiency in this ration may be responsible for poor growth, although experiments are in progress which indicate that the action of gelatin in these rations may be influential in creating an increased dietary requirement for tryptophane. Since relatively good growth is obtained on a ration which contains only 9% casein when dextrin or cornstarch is employed as the carbohydrate one must consider an alteration of the dietary requirement of amino acids, other than tryptophane, which are low in this ration. It appears further that the amino acid requirements as elaborated by Rose ('37) are subject to modification dependant on factors such as those described above.

For example the dietary tryptophane requirement of the growing rat seems to approximate 150 mg.% whereas Rose sets 200 mg.% as the minimum value. In this particular we are aware however, that although the tryptophane values used in the calculations are taken from the best accepted literature values, some of them may be in error.

Although the tryptophane deficiency encountered in the present study is sufficient to impair growth, the severe symptoms reported by Albanese ('45) such as corneal vascularization and cataract were not seen.

The observations made here indicate that a diet cannot be correctly and completely evaluated on the basis of its known chemical entities alone. Quantitative as well as qualitative relationships can be obscured by alteration of experimental conditions and interplay of nutrients. It is further evident that there is an increasing need for more information concerning the relationships between animal and bacterial nutrition.

#### SUMMARY

The addition of corn or corn grits to a nicotinic acid-low synthetic ration results in a profound growth retardation which can be completely counteracted by the inclusion of from 1-1.5 mg. of nicotinic acid or 50 mg. of tryptophane per 100 gm. of ration. Other cereals containing less nicotinic acid produce no such effect.

The kind of carbohydrate and the level of tryptophane influence the extent of the undesirable effect of corn. Glucose, dextrin and lactose were beneficial in their action. Small amounts of milk were also helpful in counteracting the growth depression but further benefits resulted from the addition of nicotinic acid. A low level of vitamins accentuated the deleterious action of corn.

Nicotinamide and ethyl nicotinate were as active as nicotinic acid on an equal molar basis while nicotinamidemethochloride, trigonelline, and N-phenylnicotinamide were inactive.

Non-corn rations low in tryptophane and nicotinic acid also gave poor growth when sucrose was used as the carbohydrate. The addition of tryptophane or nicotinic acid or the use of a carbohydrate which produced a favorable intestinal flora gave growth results comparable to those obtained with corn supplemented rations.

The factors affecting the dietary tryptophane requirement and the importance of a desirable intestinal flora in this relationship are discussed.

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# THE RELATION OF THE AMOUNT OF THIAMINE IN THE RATION OF THE HOG TO THE THIAMINE AND RIBOFLAVIN CONTENT OF THE TISSUE <sup>1,2</sup>

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## TWO FIGURES

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Hughes ('41); Miller et al. ('43); Ensminger et al. ('43); and Ellis and Madsen ('44) have reported that the thiamine content of pork tissue is related to the amount of thiamine ingested by the pig. This means that pork may be enriched with extra thiamine by the use of feeds high in vitamin B<sub>1</sub>. A program advocating the enrichment of pork by feeding swine rations high in this vitamin would increase the supply of thiamine in the American dietary.

Pork is one of the richest, naturally occurring sources of thiamine. It is eight times, and frequently higher in thiamine than beef according to values reported by Kemmerer and

<sup>1</sup> Published as Scientific Paper no. 647, College of Agriculture and Agricultural Experiment Stations, State College of Washington, Pullman. This report is from a thesis submitted by the senior author in partial fulfillment of the requirements for the degree of Master of Science. The investigations reported herein are a continuation of work initiated by Ensminger et al. ('42).

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Steenbock ('33); Waisman and Elvehjem ('41); and Richardson et al. ('44). According to values published by Waisman and Elvehjem ('41), pork is three to four times higher in thiamine than lamb. Therefore, if meat is to be enriched in thiamine, it follows that pork should logically be used since the pig is much more efficient than other meat animals in depositing the thiamine of the feed in its tissues.

The present investigation was undertaken in an effort to obtain additional information on the relationship of thiamine in the ration of the hog to the amount contained in the tissue. In addition, information was obtained on (1) the distribution of thiamine and riboflavin in various pork tissues, including the ham, loin, shoulder, liver, heart, and kidneys, (2) the efficiency of vitamin B<sub>1</sub> utilization, (3) the difference in the intestinal flora of pigs fed natural grain rations as compared to purified rations, (4) the changes in thiamine content of pigs of different ages by slaughtering animals at birth, at weaning, and at the end of the experimental feeding period, and (5) the changes in thiamine content of the hams of pigs obtained by performing biopsies on the biceps femoris at 3-week intervals during the growth period.

#### EXPERIMENTAL

Results obtained from preliminary feeding trials conducted in 1942 and 1943 were used as a basis for conducting these trials in the summer of 1944. The work reported herein confirms the results obtained in 1942 and 1943. In the 1944 trials, twenty pigs were divided into the five lots shown in table 1. The pigs were distributed equally in the several lots with respect to sex, weight, and age. The rations fed are shown in table 1.

A modification of the "paired-feeding technique" (Mitchell and Beadles, '30), was used in order to keep the feed intake constant among animals within the purified ration group (lots I, II, and III) and within the natural grain ration group (lots IV and V). Thus, in the purified ration lots, the feed consumption of the other nine pigs was limited to the amount consumed

TABLE 1  
*Rations fed to the five lots of pigs on the feeding trials.*

GROUP LOT	A				B			
	I	II	III	IV	V			
NO. OF PIGS	2	4	4	4	4			
RATION TYPE	PURIFIED				NATURAL GRAIN			
Added thia- mine or thia- mine content of feed	None	0.19 mg. daily per kg. live weight	0.42 mg. daily per kg. live weight	0.11 mg. daily per kg. live weight (con- tained in feed)	0.21 mg. daily per kg. live weight (con- tained in feed)			
The basic ration for all pigs in lots I, II, and III:								
Purified ration:	Vitamin supplements (amounts daily per kg. live weight):				Wheat	46.0%	Wheat	36.0%
Casein <sup>1</sup>	26.1%	Riboflavin	0.12 mg.	Tankage	Barley	35.0	Barley	35.0
Sucrose	57.7	Niacin	1.20 mg.	Alfalfa	Tankage	13.5	Tankage	7.0
Lard	11.0	Pyridoxine	0.20 mg.	Salt	Wheat germ meal	5.0	Wheat germ meal	15.5
Salt mix <sup>2</sup>	5.2	Choline	10.00 mg.		Alfalfa	0.5	Alfalfa	5.0
	100.0%	Calcium pantothenate	0.50 mg.		Oyster shell	100.0%	Oyster shell	1.0
Plus 6.2% oat hulls treated to destroy thiamine <sup>3</sup> (added to supply bulk)		Shark liver oil <sup>4</sup>	167.00 mg.		Salt	0.5	Salt	100.0%
		Vioosterol	67.00 mg.					
		Vitamin E <sup>5</sup>	50.00 mg.					
		Vitamin K <sup>6</sup>	2.00 mg.					

<sup>1</sup> Purified casein obtained through cooperation of Mr. G. D. Turnbow, Vice President and General Manager, Golen State Co., Ltd., San Francisco, California.

<sup>2</sup> Salt mix:

Potassium chloride	6.7%	Calcium carbonate	16.9	Iron pyrophosphate	2.100%	Zinc oxide	0.016
Dicalcium phosphate	46.3	Magnesium carbonate	8.5	Manganese chloride	0.020	Sodium carbonate	0.016
Sodium chloride	13.5	Potassium phosphate	5.7	Copper sulfate	0.020	Potassium iodide	0.237
							100.000%

<sup>3</sup> Acknowledgment is made of the cooperation extended by Mr. E. M. Williams, Manager, Livestock Chow Sales, Ralston Purina Company, St. Louis, Missouri, in supplying the oat hulls treated to destroy thiamine.

<sup>4</sup> Bio-Vita IONA, Natural Shark Liver Oil Blend containing 10,000 units vitamin A per gram. Kindly supplied by Bio-products, Inc., Astoria, Oregon.

<sup>5</sup> Supplied through courtesy of Lederle Laboratories, Pearl River, New York.

by the pig with the smallest appetite — with the quantity of thiamine being the only variable among the lots of pigs. The level of thiamine was controlled by adding definite amounts of the crystalline vitamin to the ration fed the pigs in lots II and III in the purified ration group. Likewise, the eight pigs fed the natural grain rations were considered as a group, with the feed intake of the other seven pigs limited by the pig consuming the least amount of feed. In these lots, the amount of thiamine was controlled by feeding natural grain rations composed of feeds containing different amounts of thiamine. All pigs were fed individually and promptly at three definite feeding periods daily — 6 A.M., 1 P.M., and 8 P.M.

The pigs were kept on raised floors in order to prevent coprophagy and were allowed outside as well as inside runs. The raised floors were removed and thoroughly washed three times daily as a further precaution to prevent the animals from consuming their feces.

In order to prevent the development of rancidity, the purified ration was kept in a refrigerator and not more than 2 days' feed requirements were mixed at one time. Refused feed was discarded after weighing and the individual troughs were cleaned and washed following each feeding.

The six B-complex vitamins fed (thiamine, riboflavin, niacin, pyridoxine, calcium pantothenate, and choline) were dissolved in 20% ethanol solutions and stored in a refrigerator. Vitamins A and D were kept separately, whereas E and K were mixed together. Two days' requirements of the B-complex vitamins and 3 days' requirements of the fat-soluble vitamins were fed every second and third day, respectively. The amounts were measured in calibrated pipettes and placed on a small amount of the feed on the top of the ration at feeding time. In this manner, struggling with the animals was avoided and complete consumption of the vitamins was obtained.

All the pigs were slaughtered when those on the thiamine deficient ration ceased to make any further nutritional progress. This point was reached when the pigs receiving no sup-

plement of thiamine lost their appetites and began to lose weight.

In order to provide a record of the thiamine content of the body tissues at various stages of development, two pigs of similar breeding to those fed on experiment were slaughtered at birth and two at weaning. In addition, one extra pig was fed in each of lots IV and V for the purpose of removing muscle sections from the biceps femoris at 3-week intervals during the experimental period. These samples were analyzed for thiamine content and used as a basis for comparison with samples from the pigs fed throughout the experimental period.

The comparative slaughter technique was employed in order to determine changes in thiamine content of several tissues from birth to weaning and from weaning to the end of the 56-day feeding period.

Following slaughter of the experimental animals, the carcasses were chilled for 24 hours at 33°F. Samples were taken within 24 to 48 hours following slaughter and were frozen immediately. They were stored at 0°F. for 30 days, during which time all the analyses were made. Ham, loin, shoulder, and kidney samples were taken from the right side of each carcass. After all extraneous fat was removed from these samples they were finely ground. The ground meat from each cut was thoroughly mixed before a sample was taken for assay. The left half of each carcass was skinned, boned, and ground together with the fat from that side, in order to secure a composite "sausage" sample.

Thiamine and riboflavin were determined by the thiochrome method, using the procedure developed by Conner and Straub ('41) for the combined determination of thiamine and riboflavin. The only modification of this method was in the manner of preparing the sample. This involved weighing an amount of the previously ground fresh or frozen sample and mixing in a Waring Blendor with the desired volume of extractant. Aliquots were obtained and transferred to volumetric flasks.

Samples for the determination of fat and moisture were removed from the bottles of frozen meat and weighed at the

same time as those taken for the vitamin analyses. The method described by Pitman ('32) was used to determine moisture and fat in the "sausage" samples, whereas slight modifications were made of the A.O.A.C. ('40) method for the determination of fat and moisture in the other samples. The drying time for the moisture determination was extended an additional 3 hours on all samples. The extraction of fat from the liver samples was extended to 48 hours.

#### EXPERIMENTAL RESULTS AND DISCUSSION

A record of the feed consumption and body weight gains is given in table 2. Some differences were observed among lots of pigs in average daily gains made over the 8-week feed-

TABLE 2

*Record of feed consumed and gains in weight by the pigs on the feeding trials.*

GROUP	A			B	
LOT	I	II	III	IV	V
RATION	PURIFIED			NATURAL GRAIN	
	No thiamine	0.19 mg thiamine daily per kg. live weight	0.42 mg thiamine daily per kg. live weight	0.11 mg thiamine daily per kg. live weight (contained in feed)	0.21 mg. thiamine daily per kg. live weight (contained in feed)
Number of pigs	2	4	4	4	4
Length of feeding period (days)	56	56	56	56	56
Average initial weight (pounds)	41.5	41.8	41.7	45.2	43.5
Average final weight (pounds)	70.5	80.2	80.5	78.8	84.0
Average total gain (pounds)	29.0	38.4	38.8	34.6	40.5
Average daily gain (pounds)	0.52	0.68	0.69	0.62	0.72
Average daily ration (pounds)	1.77	1.77	1.77	2.16	2.16
Feed per cwt. gain (pounds)	341	258	255	349	298

ing period. The pigs fed the thiamine deficient ration gained at a slower rate than the pigs fed 0.19 mg. thiamine daily per kilogram body weight. Increasing the thiamine intake from 0.19 to 0.42 mg. daily per kilogram body weight had no significant effect on the average daily gains or efficiency of feed utilization. Similar differences in body weight gains were noted between lots IV and V. Since the ration fed in lot V included an additional ingredient (wheat germ meal) as compared with the ration fed in lot IV, the increased gains cannot be attributed alone to the additional thiamine in the ration because the wheat germ meal may have contributed other factors which may have influenced body gains and efficiency of feed utilization.

Of much importance is the finding that the pigs on the thiamine deficient ration required 83 to 86 lbs. more feed, respectively, per 100 lbs. gain than the pigs in lots II and III which were fed the same ration plus different levels of thiamine. Since the pigs on the thiamine deficient ration ate the same amount of feed as the pigs in lots II and III, and still required much more feed per 100 lbs. gain, this definitely shows that thiamine is concerned with the efficiency of feed utilization. This is very logical since thiamine is involved in carbohydrate metabolism. Further evidence to demonstrate the increased efficiency of feed utilization by the pigs fed either the purified ration plus thiamine or the natural grain ration is shown by the percentages of body fat in the animals of each lot. The carcasses of the pigs receiving the purified ration without thiamine contained only 21% fat. The carcasses of those receiving either the purified ration plus thiamine or the natural grain ration all contained between 25 and 27% fat.

Essentially the same type of symptoms as those recorded by Van Etten et al. ('40) and Ellis and Madsen ('44) resulted from the feeding of the thiamine deficient ration. Vomiting was the first symptom observed in these pigs fed a thiamine deficient ration. It was first observed and occurred frequently after the animals had been on the thiamine deficient ration for 33 days. Appetite started to decrease during the latter



TABLE 3  
Average thiamine content of tissues<sup>1</sup> (fresh, and dry, fat-free bases) from pigs fed different levels of thiamine.

LOT	RATION	NO. OF PIGS	BASES OF THIAMINE CONTENT	SAUSAGE	HAM	LOIN	SHOULDER	HEART	LIVER	KIDNEY
I	Purified no thiamine added	2	Fresh	.24	1.64	1.28	.52	.79	.86	.89
			Dry, fat-free	1.43	7.33	5.56	2.41	4.12	3.48	4.30
II	Purified 0.19 mg. thiamine daily per kg. body weight	4	Fresh	3.66	8.54	8.74	7.29	5.58	4.95	4.73
			Dry, fat-free	29.17	38.34	38.86	32.10	28.86	18.17	26.17
III	Purified 0.42 mg. thiamine daily per kg. body weight	4	Fresh	5.64	10.65	11.34	9.38	6.11	4.37	5.26
			Dry, fat-free	37.73	41.32	49.42	41.08	30.79	16.32	33.24
IV	Natural Grain 0.11 mg. thiamine daily per kg. body weight (contained in feed)	4	Fresh	4.55	10.36	10.29	7.07	4.58	4.03	3.20
			Dry, fat-free	31.29	45.23	43.12	32.98	24.77	15.06	17.71
V	Natural Grain 0.21 mg. thiamine daily per kg. body weight (contained in feed)	4	Fresh	5.67	11.73	12.69	6.76	4.76	4.03	4.00
			Dry, fat-free	36.59	51.86	52.46	43.66	26.53	14.48	22.59

<sup>1</sup>Micrograms per gram.

half of the experimental feeding period, and was followed closely by a corresponding retardation of growth. Ellis and Madsen ('44) observed that failing appetite usually was the first sign of thiamine deprivation. In the 1943 and 1944 work at this station, vomiting occurred and was followed in a day or two by a decreased appetite. A slight staggering gait was noted in the thiamine deficient pigs during the final 5 days of these trials. On the fifty-third day one of these pigs showed signs of muscular incoordination when it trembled and fell

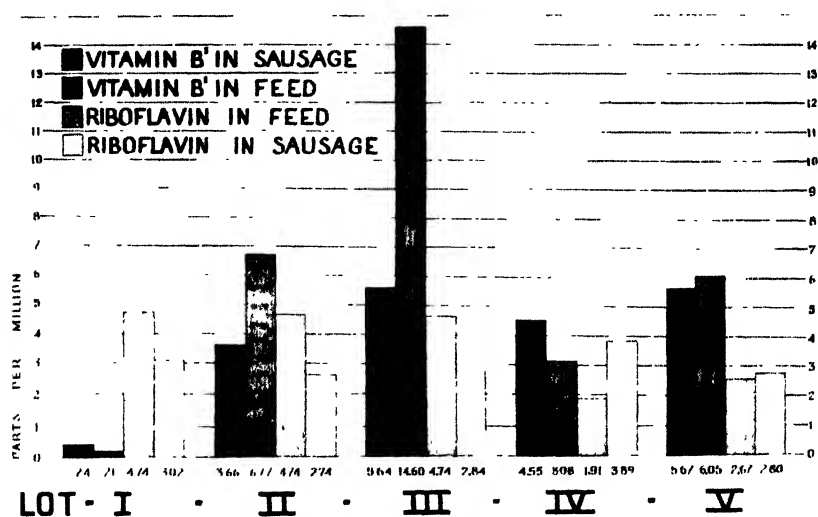


Fig. 1 The relationship of thiamine and riboflavin content of swine rations to the amount of these vitamins deposited in the pork tissues.

while eating. After several unsuccessful attempts to regain a standing position, the pig was finally able to stand without further indications of muscular incoordination.

Data showing the average thiamine content of the pork are presented in table 3 and figure 1. The values in table 3 show that the ham and loin have a consistently higher level of thiamine than the other tissues. Generally, the order of the tissues in thiamine content from highest to lowest is as follows: loin and ham, shoulder, heart, liver, and kidney. The thiamine content

of the composite carcass sample ("sausage") made from the left half of each carcass is intermediate between the level of thiamine in the heart and liver. The thiamine content of the "sausage" is a good indication of the relative level of this vitamin in the ration (fig. 1).

The thiamine values shown in table 3 indicate that generally the pigs fed natural grain rations were much more efficient in depositing the thiamine in the ration in their tissues. For example, ham samples from pigs in lots III and IV, show that the pigs in lot IV fed the natural grain ration received about one-fourth as much vitamin B<sub>1</sub> as those fed the purified ration in lot III. Yet, the pigs in lot IV had about as much thiamine deposited in their hams as those pigs fed approximately four times as much thiamine in lot III (purified ration). This difference in favor of natural grain rations, might possibly be due to the fact that thiamine in natural grains is released more slowly and is therefore more efficiently utilized. The manner in which the synthetic thiamine was fed and the ease of excretion of the excess vitamin may have contributed to less efficiency of utilization.

Our findings with respect to the higher thiamine content of pork skeletal muscle as compared to liver coincide with those of Miller et al. ('43). The liver is apparently the least responsive to the effect of added thiamine in the ration. The kidney and heart are more responsive than the liver to added dietary thiamine, but not nearly as much as the skeletal muscles.

Statistically, the difference between ration groups shown in table 3 was highly significant on both the fresh and dry, fat-free bases. However, the greater portion of the difference was due to the low values obtained for the pigs in lot I, which were fed the thiamine deficient ration. On the other hand, the variation among pigs within lots was large, indicating that great variability exists among pigs given the same treatment. As Miller et al. ('43) have indicated, this is further evidence to explain some of the wide variations in thiamine values for pork which have been reported from time to time.

Using the comparative-slaughter technique, it was shown that the thiamine content of pork, on a fresh basis, increased from birth to weaning and decreased gradually from weaning to the end of the 56-day feeding period. Data showing these values are presented in table 4. No explanation is known for the marked increase in thiamine from birth to weaning. A possible explanation might be that the pig is receiving a high fat diet (on a dry-matter basis) while suckling its mother, and as a result needs less thiamine and hence deposits more in the tissue. Ellis and Madsen ('44) have shown that fat lessens the pig's need for thiamine.

TABLE 4

*Changes in thiamine content (fresh basis) from birth to weaning, and weaning to the end of the 56-day feeding period in six pork cuts.*

Pigs	TISSUE SAMPLE					
	Ham	Loin	Shoulder	Kidney	Heart	Liver
Birth (2 pigs)	5.65	8.30	9.42	5.52	8.84	5.98
Weaning (2 pigs)	20.17	18.62	16.02	9.52	14.82	17.02
After 56-day feeding period (ave. experimental pigs)	8.58	8.87	6.20	3.61	4.36	3.65

<sup>1</sup> Micrograms per gram.

Observations in this study indicate that true storage of thiamine does occur in the pig and that the pig uses stored thiamine. At the beginning of the experimental feeding period, the analyses of two animals representative of those used in the experiment, showed that each pig contained approximately 200 mg. of thiamine in its entire carcass (exclusive of the usual offal) at the start of the experiment. At the end of the 56-day feeding period, those animals fed the thiamine deficient ration still contained slightly over 77 mg. of thiamine in their entire carcass (exclusive of offal). Since the purified ration supplied approximately 0.16 mg. of thiamine daily to each of these animals, it is reasonable to assume

that these animals were using stored thiamine at the rate of 2.21 mg. per animal daily, or 0.09 mg. daily per kilogram of body weight. Certainly there was no dietary supply of thiamine which could have supplied these animals with the 77 mg. of thiamine which remained in their bodies at the end of the 56-day depletion period. Another reason for stating that storage of thiamine occurs and that the pig can utilize this stored thiamine for long periods of time, is the fact that it took 33 days before the appetites of these animals were affected and 56 days before their appetites were lost completely.

TABLE 5

*Changes in thiamine content of ham (biceps femoris muscle) during the suckling and early feeding period of pigs.*

AGE	THIAMINE CONTENT OF HAM (FRESH BASIS) <sup>1</sup>
At birth (2 pigs)	5.65
At weaning (2 pigs)	20.17
At start of feeding period (2 pigs on grain ration) <sup>2</sup>	9.97
After 23 days of feeding (2 pigs on grain ration) <sup>2</sup>	7.48
After 44 days of feeding (2 pigs on grain ration) <sup>2</sup>	6.50

<sup>1</sup> Micrograms per gram.

<sup>2</sup> Determined on same two pigs by performing biopsies.

Muscle sections of the hams of live pigs taken <sup>4</sup> at 3-week intervals and analyzed for thiamine show that the thiamine content of the ham (biceps femoris) increased from birth to weaning and gradually decreased after weaning to the forty-fourth day of the experiment period, at which time the last ham sample was taken. Changes in water and fat content of the tissues from birth to weaning and later stages may account for a part of the change in thiamine content during this period. However, it is believed that the greater-portion of the change in thiamine content of the ham muscle may be attributed to other causes. These data, presented in tables 4 and 5, were obtained from assays made of ham tissues from two new-born

<sup>4</sup> Obtained by Dr. P. J. Pfarr, Assistant Professor of Veterinary Anatomy, and Dr. E. C. McCulloch, Research Veterinarian.

pigs, two pigs at weaning time, and the two extra pigs on the feeding experiments from which live ham muscle (biceps femoris) samples were taken at 3-week intervals. Samples were taken alternately from the left and right leg so that there was a 6-week interval before a second biopsy was performed on the same leg. These biopsies were performed three times with no apparent harmful effect or interference with the locomotion of the two pigs.

Average riboflavin values for the tissues from the pigs slaughtered at the termination of the experiment are shown in table 6. All lots of pigs fed the purified ration received the same level of riboflavin whereas the two lots of pigs fed the natural grain rations received slightly differing amounts of riboflavin. However, no significant difference was noticed in riboflavin deposition among lots of pigs, indicating that the dietary level of riboflavin has much less effect on skeletal muscle tissue content of this vitamin than is true in the case of thiamine. Also, pork skeletal muscle evidently contains much less riboflavin than thiamine. However, pork heart, liver, and kidney contain more riboflavin than the skeletal muscles and more riboflavin than thiamine.

The riboflavin values shown in table 6 indicate that pigs fed natural grain rations were more efficient in depositing the riboflavin in the ration in their tissues. For example, the composite carcass samples from pigs in lots III and IV, show that the pigs in lot IV, fed the natural grain ration, received  $\frac{1}{2}$  as much riboflavin as those fed the purified ration in lot III. Yet, the pigs in lot IV had even higher levels of riboflavin deposited in the sausage than those pigs fed the purified ration with higher levels of riboflavin.

Results of this work indicate that a thiamine deficient ration fed to pigs has some effect on increased riboflavin deposition in the heart and liver. Data in table 6 show that the hearts and livers of the pigs fed the thiamine deficient ration contained slightly more riboflavin than the hearts and livers of pigs fed adequate levels of thiamine. This finding is in agreement with those of Singher et al. ('44) and Luecke et al. ('44),

TABLE 6

*Average riboflavin content of tissues (on fresh and on dry, fat-free bases) from pigs on different levels of thiamine and riboflavin intake. All values are micrograms per gram of tissue.*

LOT	DATION	NO. OF PIGS	BASES OF THIAMINE CONTENT	SAUSAGE	HAM	LOIN	SHOULDER	HEART	LIVER	KIDNEY
I	No thiamine; 0.12 mg. riboflavin daily per kg. body weight	2	Fresh	3.02	3.68	3.00	3.62	14.73	23.88	16.49
			Dry, fat-free	18.38	16.36	12.70	16.72	76.62	96.26	80.81
II	0.19 mg. thiamine daily per kg. body weight; 0.12 mg. riboflavin daily per kg. body weight	4	Fresh	2.73	4.54	3.32	4.19	13.41	22.88	10.83
			Dry, fat-free	17.65	20.69	14.82	18.69	69.48	85.45	60.98
III	0.42 mg. thiamine daily per kg. body weight; 0.12 mg. riboflavin daily per kg. body weight	4	Fresh	2.84	3.35	2.61	2.74	14.22	23.67	15.91
			Dry, fat-free	18.97	15.28	11.36	12.03	71.71	88.02	86.47
IV	0.11 mg. thiamine daily per kg. body weight; 0.06 mg. riboflavin daily per kg. body weight (contained in feed)	4	Fresh	3.89	2.81	2.41	2.57	11.45	22.27	15.37
			Dry, fat-free	19.45	12.98	10.37	12.00	61.86	82.97	85.17
V	0.21 mg. thiamine daily per kg. body weight; 0.09 mg. riboflavin daily per kg. body weight (contained in feed)	4	Fresh	2.80	3.07	2.93	2.96	11.79	19.76	19.04
			Dry, fat-free	17.91	13.63	12.20	13.26	65.74	71.20	105.56

who reported that a thiamine deficiency results in an increased riboflavin concentration in the liver of rats. A possible explanation for the increased riboflavin concentration in the liver and heart of the pig may be that with a thiamine deficiency there is a slowing of body metabolism and as a result less riboflavin is needed. Hence, the riboflavin accumulates in the liver and heart which are the main storage organs for this vitamin.

Observations were made of the rate of heartbeat, rate of respiration, and rectal temperature throughout the experiment. A slowing of the heartbeat and respiratory rate in the thiamine-deficient animals was noted during the last week preceding the end of the experiment. Rectal temperatures of these animals dropped during the last 4 days to slightly below 99°F. Upon slaughtering, enlarged hearts were found in the animals fed the purified, thiamine deficient ration. The hearts of the two thiamine deficient pigs were 54 and 91% heavier than the average of the hearts of the other pigs on this experiment. Pictures of representative hearts of a thiamine deficient pig as compared to the heart of one of the pigs which received an adequate level of thiamine are shown in figure 2.

The bactericidal action of the blood sera of the pigs against *Escherichia coli*, *Salmonella typhimurium*, *Eberthella typhosa*, and *Staphylococcus aureus* did not appear to be influenced by type of diet or thiamine level. Coliform counts of the feces, on a wet basis, of the pigs receiving purified rations, either without thiamine or at different levels of thiamine, were approximately 1000 times higher than the coliform counts, very largely *Escherichia coli*, of the feces of pigs receiving natural grain rations. This suggests a possible difference in intestinal vitamin synthesis between pigs fed a purified as compared to a natural grain ration, since Gant et al. ('43) obtained indications that *Escherichia coli* is the organism mainly responsible for the synthesis of biotin and folic acid or related factors. Work by other investigators substantiates this possibility. Cunha et al. ('43) demonstrated that rats on a basal ration composed largely of corn (natural grain ration) re-



ceived an insufficient supply of the norite eluate fraction (a source of folic acid, vitamins B<sub>10</sub> and B<sub>11</sub>). Such a deficiency can ordinarily be produced with rats receiving a purified ration only when a sulfa drug is added, as was demonstrated by Black et al. ('41). This indicates that corn, or other constituents of a natural grain ration, in some manner, decreases intestinal synthesis of certain vitamins. This has recently

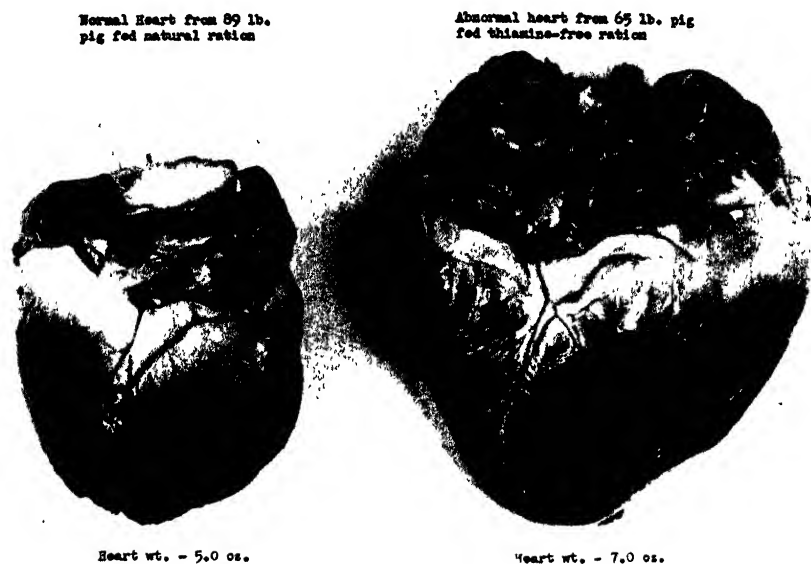


Fig. 2 The heart at left is from a pig fed the purified ration with adequate thiamine. The heart at the right is that of a pig fed the same ration without thiamine. Live weights of the two pigs were 89 and 65 lbs., respectively.

been confirmed with the chick and rat. Briggs et al. ('45) showed that when corn meal was substituted for part of the dextrin in a purified ration, growth, mortality, and hemoglobin formation of chicks on the basal ration was poorer. Krehl et al. ('45) showed that a growth retardation was caused in rats when 40% of corn grits was included in a low protein ration. All of this work strongly indicates that there may be a difference in intestinal vitamin synthesis by animals fed natural grain rations as compared to purified rations.

## SUMMARY AND CONCLUSIONS

The poorest gains were made by pigs on the thiamine deficient ration. Likewise, the pigs on the thiamine deficient ration required much more feed per pound of gain, definitely showing that thiamine is concerned with the efficiency of feed utilization by the pig.

Efficiency of utilization of the thiamine and riboflavin in the ration was much greater for pigs fed the natural grain rations than for those fed the purified rations.

A positive relationship exists between thiamine intake and the deposition of this vitamin in the tissues of hogs. This makes it possible to increase the content of thiamine in pork by using feeds high in this vitamin. Usually the loin and ham are highest in thiamine content, followed by the other cuts in the following order: shoulder, heart, liver, and kidney.

Pork liver is higher in riboflavin content than the other pork cuts, followed by the kidney, heart, ham, shoulder, and loin. A thiamine deficiency had no lowering effect on the deposition of riboflavin in the skeletal muscle tissues. There was a slight increased concentration of riboflavin in the liver and heart of the pigs on a thiamine deficient ration as compared to the pigs on a ration containing adequate thiamine.

Symptoms observed on the thiamine deficient pigs were vomiting, anorexia, slight staggering, cyanosis, and a reduction in rectal temperature, heart beat and respiration during the final stages of the experiment. Enlarged hearts were obtained from the pigs on the thiamine deficient ration.

Coliform counts of the feces of pigs fed the purified ration, with or without thiamine, were approximately 1000 times higher than for pigs fed natural grain rations. This may suggest a possible difference in intestinal vitamin synthesis between pigs fed natural grain rations as compared to purified rations. Further evidence for this possibility is presented and discussed.

Data were obtained to show that storage of thiamine occurs and that the pig can utilize stored thiamine over a long

period of time. Evidence for this is that 56 days were required for the pigs to lose their appetites after being placed on a thiamine deficient ration.

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# FURTHER STUDIES ON THE CALCIUM REQUIREMENT OF PRESCHOOL CHILDREN <sup>1, 2</sup>

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## TWO FIGURES

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## INTRODUCTION

In 1939 this laboratory published a report dealing with the calcium requirements of five girls, from 3 to 5 years old (Outhouse, Kinsman, Sheldon, Twomey, Smith and Mitchell, '39). At that time the authors stated that these well-nourished children needed less than 1 quart of milk daily for the satisfaction of their calcium needs. The present paper, dealing with a similar investigation of preschool boys, presents a similar conclusion; only one of the seven subjects needed as much as 1 pint of milk daily and he could have satisfied his individual requirement for calcium by ingesting, in addition to an otherwise satisfactory diet, 720 gm. of milk daily.

## EXPERIMENTAL

The children used in this study were seven normal, healthy boys ranging in age from 2.7 to 5.8 years at the beginning of the experiment. A description of their height-weight-age

<sup>1</sup> A preliminary report of these data was made before the Amercain Home Economics Association at its annual meeting in Pittsburgh, June, 1939.

<sup>2</sup> Aided by a grant from the American Dry Milk Institute, Inc., Chicago.

relationships in comparison with the standards of Grandprey ('33) has been presented in a former publication (Kinsman, Sheldon, Jensen, Bernds, Outhouse and Mitchell, '39). Four of the subjects (R, C, Br and Jw) were presumably well nourished in respect to calcium since they had been accustomed not only to drinking liberal quantities of milk at each meal but also to having for one meal each day either bread and milk or a cream soup. The dietary history of the other three boys was unknown. Subject D was brought from another children's home expressly for the experiment and W and G, brothers, because of unsatisfactory home care were admitted to the Cunningham Children's Home on the day on which the experiment started.

A description of the experimental conditions under which the subjects were held, as well as the analytical methods used, may be found in the publication of Kinsman et al. ('39) and of Outhouse et al. ('39). Only the important features of the regimen need be repeated here: (a) a daily allowance of approximately 1000 international units of vitamin D was fed as cod-liver oil; (b) the low-calcium content (i.e., 339 mg. daily) of the basal dietary was purposely achieved by limiting the amounts of vegetables to only one or two servings daily and the quantity of milk solids to the equivalent of approximately 200 gm. of fluid milk daily; (c) the basal dietary which was fed throughout the study was supplemented during periods I, III, IV and V by non-fat dry milk solids<sup>3</sup> which brought the average intakes of calcium to 1600, 555, 704, and 904 mg., respectively, during these periods; (d) the metabolic periods were 7 days in length and period I to V, respectively, lasted 91,<sup>4</sup> 49, 35, 28 and 63 days.

The method for computing the calcium requirement makes use not only of data for calcium retentions during periods I

<sup>3</sup> The calcium content of the non-fat dry milk solids ranged from 12.3 to 13.5 mg. per gram. (In two former publications, an error in the decimal place was made in reporting the calcium content of the milk solids.)

<sup>4</sup> Subject D was kept at this high level for 126 days.

to V<sup>6</sup> but also of the previously reported data (Kinsman et al., '39) concerning the extent to which each subject utilized the calcium of milk. The retentions of calcium during the period of generous calcium intake, i.e., period I, were compared with the balances during periods II, III, IV and V to determine if like retentions had been observed at lower levels of intake. In those instances in which it was reasonable to suspect that equally high retentions of calcium might have been observed at a significantly lower level of intake, the calcium requirement was obtained by use of the following formula:

$$\frac{\text{Maximum calcium retention in mg.}}{\text{Per cent utilization of milk calcium}} \times 100 = \text{Calcium requirement in mg.}$$

## RESULTS

The skeletal growth of the subjects is shown in figure 1 in comparison with the growth of boys from families above the average in economic and educational status (Simmons, '44). Five of the boys had horizontal lengths shorter than the mean for Simmons' group. However, in relation to the mean rate of growth of the latter, the rates of growth of the 7 subjects during the total experimental period, from 7 to 18 months for the various subjects, were as follows: that of subject D was considerably faster whereas those of R, W, G and JW were parallel, and those of C and Br were probably somewhat slower. For the period under discussion in this paper; i.e., from the fifth<sup>6</sup> to the thirteenth week, the growth in length of the body was satisfactory for all subjects with the exception

<sup>6</sup>Detailed data concerning the weekly intake and the urinary and fecal excretions of calcium for these seven boys and for the five girls previously studied will appear in a forthcoming publication by I. G. Macy entitled "Nutrition and Chemical Growth in Childhood," vol. II. Original Data. Charles Thomas Press, Springfield, Ill.

<sup>7</sup>The first measurements on all subjects except D were taken on the thirty-fourth day of the experiment; hence that portion of the curves referring to period I is represented by the first 45 days. D's measurements were taken at the beginning of the study.



of W whose increment during this short period only appeared to be smaller than the mean for children of his age.<sup>7</sup>

The calcium retentions of the subjects during the period when they were receiving 1600 mg. of calcium daily are recorded in figure 2 in terms of the cumulated retentions. In the lower set of curves data are given for the entire period. Considerable variability in the week-to-week balances of a given

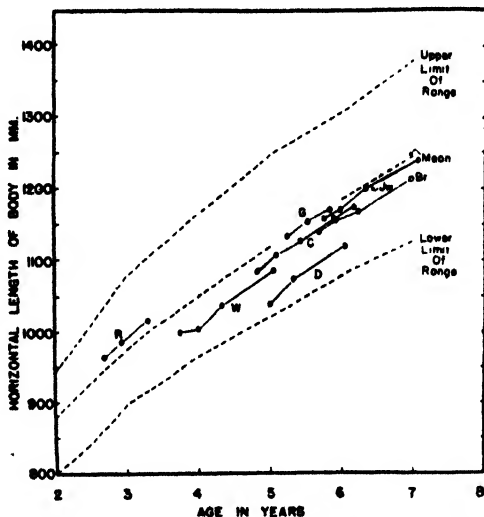


Fig. 1 The growth in horizontal length of the seven boys, R, W, C, D, G, Jw and Br, is shown here in comparison with the mean and the upper and lower limits of range of similar measurements for a group of boys from families above the average in economic and educational status (Simmons, '44). The first measurements were taken on the thirty-fourth day of the experiment for all subjects except D, whose measurements were taken at the beginning of the study.

subject is evident. From a cursory inspection of the curves one might conclude that the subjects differed markedly in the rate at which they stored calcium. Actually, however, four of the subjects, i.e., W, G, C and Jw, accumulated approximately the same quantity of calcium during the 12- to 13-week period. Moreover, during certain consecutive weeks the other

<sup>7</sup> The increment in acromial breadth for this period likewise was small; those for cristae and trochanteric breadth, however, were greater than the mean observed in Simmons' group.

three subjects stored calcium at the same rate as did the former four subjects. The uniformity in the accretion of calcium is clearly shown in the upper set of simplified curves<sup>a</sup> constructed from data for selected weeks, i.e., the entire period

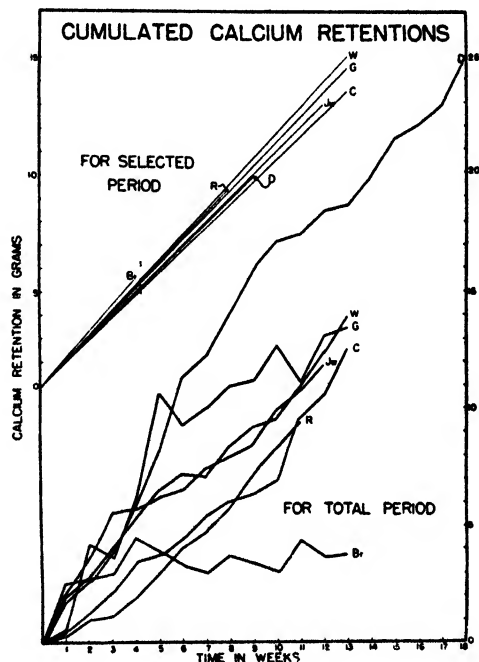


Fig. 2 The cumulated calcium retentions of the seven boys are shown (a) weekly for the entire experimental period in the lower set of curves and (b) for selected weeks in the upper set of curves which have been constructed by drawing a straight line between the zero point and the point for the total cumulated retention. The black dots recorded at 30 days in the upper set of curves refer to the cumulated calcium retentions of eight well-nourished children during the last month of a 6-month study conducted by Macy ('42).

for W, G, C and Jw, the last 9 weeks for D, the last 8 for R and the first 4 weeks for Br. For these selected weeks the daily retentions averaged 150, 154, 137, 143, 148, 161 and 143 mg., respectively, for subjects R, W, C, D, G, Br and Jw (table 1).

<sup>a</sup> In order to avoid the confusion introduced by the variable weekly balances a straight line has been drawn between the zero point and the point for total retention for each subject.

TABLE 1  
Daily calcium-requirement values and data upon which they are based.

SUBJECTS			CALCIUM RETENTION DURING PERIOD I				CALCIUM DATA DURING PERIODS OF LOWER INTAKE				PROBABLE CALCIUM REQUIREMENT		PROPOSED MILK REQUIREMENT <sup>1</sup>	
Name	Description <sup>1</sup>		For entire period	No. of weeks	For selected weeks		Period	Intake	Reten- tion	Utiliza- tion	Total	per kg.	per cm.	
	Age	Weight			Height	mg.								mg.
B	2-8	16.0	96.5	116	8	150 9.4 1.55	II	328 63		17.8	843	53	8.7	452
							III	554 94						
							IV	692 143						
							V	891 158						
W	3-9	16.4	98.9	154	13	154 9.3 1.55	II	329 68		21.3	723	44	7.3	352
							III	548 116						
							IV	703 145						
							V	899 183						
C	4-10	20.3	108.5	137	13	137 6.7 1.26	II	347 63						
							III	560 81		20.0	685	35	6.6	321
							IV	712 136						
							V	909 145						
D	5-0	16.6	103.8	197	9	143 8.6 1.36	II	350 54						
							III	504 96		17.2*	831	50	8.0	442
							IV	343 53						
G	5-3	20.4	113.2	148	13	148 7.2 1.30	II	567 83						
							III	728 98		12.7	1165	57	10.3	720
							IV	915 126						
							V	344 60						
Br	5-8	22.5	114.0	42	4	161 7.1 1.41	II	556 106		19.5	826	37	7.2	438
							III	699 119						
							IV	904 179						
							V	345 64						
Jw	5-9	20.1	116.0	143	12	143 7.1 1.23	II	545 119		17.8*	803	40	6.9	419
							III	697 110						
							IV	905 135						
							V							
Ave.				134		151 7.9 1.38					839	45	7.8	478

<sup>1</sup> Data collected at the fifth week of the study.

<sup>2</sup> Calculated on the assumption that the difference between the total requirement and that quantity (i.e., 300 mg. as an average) which preschool children could be expected to obtain from the non-milk foods of their dietary.

<sup>3</sup> Satisfactory data for the computation of per cent utilization were not obtained for this subject; hence this value refers to percentage retention.

<sup>4</sup> This value, the mean of the percentage utilization data derived from 3 periods, is used here rather than the originally reported value of 27.5 based on data for one period. This value, 17.8%, more closely agrees with the mean value for percentage retention during 4 periods; i.e., 17.7%. Moreover this subject's utilization of the calcium in  $\text{CaHPO}_4$  was only 17.3%.

The mean retention for the group was 151 mg. a value somewhat higher than the average retention of 134 mg. observed for the total, non-selected periods. On the basis of body weight and skeletal length, respectively, the above retentions are 9.4, 9.3, 6.7, 8.6, 7.2, 7.1 and 7.1 mg. per kilogram and 1.55, 1.55, 1.26, 1.36, 1.30, 1.41 and 1.23 mg. per centimeter for the children in the above sequence.

Table 1 also presents the calcium balances during subsequent periods of reduced calcium intake. In every instance the retentions of calcium were greater during period V than during period IV. This fact alone, however, should not be taken as evidence that the daily requirement for dietary calcium for all the subjects more nearly approximated 900 mg. than 700 mg. This should be obvious when it is realized that the low levels of calcium during some of the periods may not have been sufficient to allow for normal calcification of the skeletal tissues formed during that time and that consequently a portion of the calcium stored during subsequent periods of excess dietary calcium may have been used to bring those previously formed bones to a state of physiological saturation; hence the retentions during period V are not necessarily representative of the well-nourished child.

The computed requirements for dietary calcium are also given in table 1. None of the children needed 1600 mg. of calcium daily. Subject G, because of his exceedingly poor capacity to utilize calcium, apparently required as much as 1165 mg.; i.e.,  $\frac{148}{12.7\%} \times 100$ . Subject R's retention at the 891 mg. level was similar to that during period I; a requirement of 843 mg. seems probable. In placing Br's requirement at less than 900 mg., it is pertinent to mention the long period of possibly inadequate calcification; i.e., 9 weeks in period I during which very poor utilization was observed even though the daily intake approximated 1600 mg., in addition to the 16 weeks of periods II through IV; if 161 mg. represents his maximal retention of calcium under good nutritive conditions, a daily intake of 826 mg. should be sufficient for him. The requirements of D and Jw, respectively, are placed at 831 and

803 mg. solely on the basis of their retentions during period I and their probable ability to utilize calcium; unfortunately the retentions of these two subjects during periods of reduced calcium intakes showed no consistent proportionality to the intake and hence do not provide contributory evidence. Subjects W and C apparently needed a smaller quantity of calcium than did the other subjects; at intakes approximating 700 mg. their calcium retentions were similar to those during period I, hence their requirement can be placed, with reasonable assurance, at 723 and 685 mg., respectively. The mean of these proposed values for requirement for dietary calcium; i.e., 843, 723, 685, 831, 1165, 826 and 803, respectively, for subjects R, W, C, D, G, Br and Jw, is 839 mg. On the basis of weight these values become 53, 44, 35, 50, 57, 37 and 40 mg. per kilogram, whereas the percentimeter requirements are 8.7, 7.3, 6.6, 8.0, 10.3, 7.2 and 6.9 mg., listed in the same order as the values given above; the mean values for the two sets of data are 45 mg. and 7.8 mg., respectively.

#### DISCUSSION

The method used in this experiment for assessing the calcium requirements of the preschool boys involves the determination of the calcium retention during a period of generous calcium intake and its conversion into terms of dietary calcium, using data concerning the extent to which each subject could utilize milk calcium. Inherent in this procedure is the assumption that the child's only requirement for calcium is for growth; if he needed to ingest calcium to replace endogenous losses, then the total requirement would be greater than the values computed by this method. However, our investigations on children, including six of those in this study, have indicated that, at levels of intake not exceeding the requirement, no extra calcium need be provided for maintenance purposes (Kinsman et al., '39). If this is true, then the approach used in this study is not only theoretically sound but also serves as a means of determining fairly precise calcium requirements.

An important feature of this study was the feeding of generous quantities of calcium at the beginning of the study in an attempt to insure physiological calcification of previously formed, but possibly inadequately calcified bone in order to obtain subsequently, under conditions of generous calcium feeding, an accurate measure of the calcium required for growth. Some of the subjects; i.e., R, C, Br and Jw, as previously mentioned, should have been in a satisfactory state of calcium nutrition at the beginning of the study and hence their retentions during the entire 13 weeks of period I should be indicative of their day-to-day requirements for calcium. Nevertheless a selection of data was made in the case of three of these children. Subject R, following the first week of the experiment, was ill for 2 weeks; since his calcium balances during the first 3 weeks following the illness were not typical of those during the subsequent 8 weeks, they were discarded. Subject Br was virtually in calcium equilibrium during the last 9 weeks of the experiment, a response not observed in this subject even when his daily ingestion of calcium was markedly reduced as in period II. Therefore the conclusion was reached that the child's needs might be more accurately expressed by the calcium retentions during the first 4 weeks than by the mean retention of the entire 13 weeks; hence the data for the last 9 weeks were discarded. The retentions of two of the children were greater during the early weeks of the experiment than they were during the latter part, suggesting a partial inadequacy of the calcium reserves at the beginning of the experiment. In the case of D whose daily retention of calcium averaged 253 mg. during the first 9 weeks but only 143 mg. during the second 9 weeks, the data for the first 9 weeks were discarded. In the case of subject G, who retained 221 mg., as an average, during the first 6 weeks and 84 mg. during the last 7 weeks, the data for the entire 13 weeks were averaged. However, if this child was undernourished at the beginning of the experiment, the latter figure, i.e., 84 mg., doubtless would be more representative of his true requirement than would the mean value for the entire

period, namely, 147 mg. Whether or not such a selection of data as employed in the case of subjects R, D and Br is defensible is open to argument. Two results, however, have already been mentioned; namely, a greater uniformity in calcium retention among the children and a higher mean value for the group. In a group of eight children receiving 900 mg. of calcium daily for 6 months Macy ('42) observed a progressive increase in uniformity of, and in mean value for, the calcium retentions. For purposes of comparison the cumulations of calcium by her subjects during the last month of the experiment are recorded as black dots; i.e., at 30 days, in the upper set of curves in figure 2. It is obvious that the uniformity in calcium retentions, as expressed by the values assigned to the children in the present study, is as great as that observed for the children in Macy's study after 5 months on a calcium-adequate regimen.

The seven subjects in this study bring to a total of twelve the number of preschool children whose calcium metabolism has been studied in this laboratory. The daily retentions proposed for the boys average 151 mg. whereas those for the girls average 128 mg., values which, when converted to a per-kilogram basis, agree remarkably well; i.e., 7.9 and 7.8 for the boys and girls, respectively. Similar mean values can be found in the literature for preschool children who have been held on experimental diets for considerable time; i.e., 8.0 mg. per kilogram for Porter-Levin's ('32-'33, '33-'34) five children, 7.6 mg. for the five of Hawks et al. ('42), 8.1 mg. for the four reported by both Bonner et al. ('38) and Hummel et al. ('39) and 8.7 mg. for eight children of Daniels et al. ('34), the mean value for these twenty-two children being 8.2 mg. per kilogram. On the basis of a somewhat different method for selection of the calcium-balance data reported in the literature, mean values of the same order of magnitude were obtained by Duckworth and Warnock ('42), namely, 138, 145, 159 and 129 mg. daily, for 2-, 3-, 4-, and 5-year-old children, respectively.

However the quantities of calcium retained by the subjects in this study are probably higher than can be accounted for

by growth needs alone. Mitchell, Hamilton, Steggerda and Bean ('45), basing their estimate on gains in total body weight and Shohl ('39) and Holmes ('45), in attempts to take into consideration the accelerated growth of the skeletal tissues during the first year of life, have concluded that the accretion of calcium during the preschool years is very small. The daily storages during the second, third, fourth, fifth and sixth years estimated by Mitchell et al. are 105, 70, 53, 50 and 60 mg., respectively, by Shohl 110, 90, 68, 105, and 138 mg., and by Holmes 117, 91, 88, 90 and 111 mg. Of the twelve children studied in this laboratory only two retained calcium in comparable quantities; i.e., the girl, M, whose daily retentions over a 6-week period averaged 106 mg. and the boy, G, whose retentions over a 7-week period averaged 84 mg. The considerably higher accretions found for the other subjects may be the result of a calcium impoverishment of the skeletal tissues present at the beginning of the metabolic studies and resulting from inadequate calcium feeding possibly instituted at birth (Stearns, '39). If this explanation of the discrepancy between the estimated and the observed values is valid, it is obvious that a period of liberal calcium feeding considerably longer than the 13-week period used in this study would have been necessary in order to secure calcium retentions relating solely to growth.

The range in values for the proposed daily requirement for dietary calcium of the seven boys is from 685 to 1165 mg. This range is much greater than that reported for five preschool girls (Outhouse et al., '39); for their total requirement, the extremes were 615 and 665 mg. or 32 and 41 mg. per kilogram. However, by applying to the data for the girls the method of assessment herein used, slightly different requirements than those published previously can be obtained. The new figures would be, for subjects B, M, J, P and S, respectively, 725, 855, 702, 535 and 679 mg. daily; they would average 699 mg. On the basis of such values, the per kilogram requirements would be 50.0, 60.6, 39.1, 30.2 and 35.5 mg. and would average 43.1 mg., whereas the per-centimeter requirements would be 7.8,



8.7, 7.1, 4.8 and 6.4, averaging 7.0 mg. Thus the requirements of the girls on a weight or height basis show a wider range than do those of the boys. The mean values for the two groups, however, are very similar; i.e., 45 and 43 mg. per kilogram and 7.8 and 7.0 mg. per centimeter for the boys and girls, respectively.

Since the non-milk foods available to the average American child would not, in the quantities usually eaten, provide as much calcium for the physiological calcification of skeletal tissues as children under experimental conditions have retained, an expression of the milk needs of the children of this study would seem pertinent. On the assumption that they could obtain daily, from the non-milk foods currently recommended for children of this age, approximately 300 mg. of calcium, the quantities of milk with which they would need to supplement such a dietary have been calculated and recorded in table 1. The resulting milk requirements range from 321 to 720 and average 478 gm. Six of the boys needed less than 1 pint daily; one who had a poor capacity to utilize dietary calcium could not have satisfied the calcium needs assigned to him by ingesting less than  $\frac{1}{4}$  of a quart of milk.

#### SUMMARY

Seven preschool boys were subjected to a 40-week experiment during which calcium balances at different levels of calcium intake were determined for the purpose of assessing requirements for calcium. The values believed to be representative of the daily needs of the children were, in order of increasing age of the subjects, 150, 154, 137, 143, 148, 161 and 143 mg. On the basis of such retentions and at the rate at which each subject could utilize milk calcium, the daily requirements for dietary calcium have been computed, the resulting values being 843, 723, 685, 831, 1165, 826 and 803 mg. or 53, 44, 35, 50, 57, 37 and 40 mg. per kilogram, respectively. Assuming that children, under acceptable dietary conditions, could secure approximately 300 mg. of calcium daily from the non-milk foods which they eat and that such calcium is utilized

to the same extent as is that of milk, the requirements of six of the children would have been met by a milk supplement no greater than 2 cupfuls. One subject would have needed as much as  $\frac{3}{4}$  of a quart.

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# THE SIGNIFICANCE OF FATTY INFILTRATION IN THE DEVELOPMENT OF HEPATIC CIRRHOSIS DUE TO CHOLINE DEFICIENCY

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ONE TEXT FIGURE AND ONE PLATE (FIVE FIGURES)

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It is now well established that ingestion of a diet low in protein and choline will result in hepatic necrosis and cirrhosis in the rat (Gyorgy and Goldblatt, '39, '42; Webster, '41; Blumberg and McCollum, '41; Daft et al., '41). However, before this was demonstrated, Connor ('37) had expressed the view that Laennec's cirrhosis in man is the ultimate outcome of fatty infiltration of the liver and surmised that if a given cirrhotic liver is not fatty, it is only so because the fat previously present has disappeared. Since choline deficiency rapidly results in fatty infiltration of the liver it has seemed reasonable that the hepatic necrosis and cirrhosis of choline deficiency may be the obligatory consequences of chronic fatty infiltration. However, no adequate proof of this hypothesis has been offered. The present paper describes an attempt to determine experimentally the significance of the accumulation of fat in the development of the subsequent hepatic necrosis and fibrosis due to choline deficiency.

## EXPERIMENTAL

Weanling rats of the Vanderbilt strain (Wolfe et al., '38) were offered the experimental diets when they had attained

a weight of 45–50 gm. They were housed in individual cages and, with the exception of group H, were offered the various diets ad libitum. A large number of the animals died before the termination of the experimental period of 120 days. Usually this was recognized in advance and, when it was deemed that such an animal could not survive the night, it was sacrificed by decapitation and the liver excised, in the same manner used for all rats after 120 days. About half the liver was then taken for total fat analysis (Handler and Dann, '42) and the remainder was fixed in neutral 10% formalin. Sections were stained with hematoxylin and eosin.

*Optimal dietary conditions for the production of hepatic cirrhosis due to choline deficiency*

Before proceeding to examine the role of fatty infiltration it was thought necessary to first establish optimal dietary conditions for the production of hepatic cirrhosis due to choline deficiency. Of the various diets which have been used to this end, the most effective appear to have been those of Daft et al. ('41). These workers employed diets containing 4% casein, with very little fat and starch as the carbohydrate. It was, however, thought desirable to compare such a diet with others (a) using sucrose as the dietary carbohydrate since Griffith ('41) was unable to produce "hemorrhagic kidneys" when starch was the dietary carbohydrate, (b) containing greater amounts of fat and cholesterol, and (c) containing somewhat more protein since this low level permits only very slow growth. The composition of these diets and the others employed in this study are summarized in table 1.

When starch was used as the dietary carbohydrate (group A) on a low fat diet all but nine animals survived the 120-day experimental period. The livers of all this group were found to contain moderate quantities of fat and most of those which were appreciably fatty showed a slight to moderate degree of necrosis. The necrosis appeared to commence around the central vein and was usually restricted to this region. The liver cells showed various stages of necrosis, from hyaliniza-

tion or poor staining of the cell to complete disintegration. The older necrotic areas appeared as foci of loss of liver substance with replacement by masses of refractive non-staining bodies (yellow, yellow-orange, or yellow-green) lying in a minimal amount of scar tissue. This material presumably was identical with the material named "ceroid" by Lillie et al. ('42). It lay either free in scar tissue or within macrophages. The scarring was very slight. The liver cells that remained showed a marked fatty change and were grouped into roughly circular

TABLE 1  
*Percentage composition of experimental diets.*

INGREDIENT	D I E T							
	A	B	C	D	E	F	G	L
Casein	5	5	5	7	10	8	5	18
Starch	85.5		68	66	63	63		53.8
Sucrose		85.5						
Lactose							68	
Cottonseed oil	3	3	10	10	10	10	10	10
Lard			10	10	10	10	10	10
Cod liver oil	2	2	2	2	2	2	2	2
Salts <sup>1</sup>	4	4	4	4	4	4	4	4
Cystine	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Cholesterol			0.5	0.5	0.5	0.5	0.5	0.5
Nicotinamide						2		1.2

In addition, to each kilogram of diet were added the following. thiamine chloride 10 mg., riboflavin 20 mg., pyridoxine 10 mg., calcium pantothenate 100 mg., p-aminobenzoic acid 100 mg., 2-methylnaphthoquinone 5 mg.

<sup>1</sup> Hubbell, R. G., L. B. Mendel and A. J. Wakeman, 1937 J. Nutrition, vol. 14, p. 273.

islands surrounded by delicate strands of connective tissue consisting of one or two layers of fibroblasts lying next to newly formed capillaries. There was practically no inflammation except for rare tiny foci of round cells and eosinophils. There was no hemorrhage.

The diet of group B differed from that of group A only in that sucrose was the dietary carbohydrate. Of the total of twenty-four animals offered this diet, nineteen died before the end of the 120 period, the mean survival time of this group

being 76 days. Histological study was made of the livers of the survivors and the last ten rats to die. The other animals died unexpectedly and, when found, sufficient post-mortem decay had occurred to warrant discarding them.

Of the livers examined, two showed essentially no fatty change. The others were moderately fatty. However, in only two was there any evidence of necrosis and this was slight. This may have been due to the early death of these animals so that there was not sufficient time for any extensive necrosis or it may be related to the only moderate degree of fatty infiltration. The latter seems reasonable since the rats of group B ate, on the average, 5.1 gm. per day while those in group A

TABLE 2  
*Summary of histological findings in choline deficiency under various circumstances.*

GROUP	MEAN WEIGHT OF SURVIVORS	LIVER WEIGHT	LIVER FATTY ACIDS	HISTOLOGICAL EXAMINATION		
				Fat	Necrosis	Fibrosis
	<i>gm.</i>	<i>gm.</i>	<i>% wet weight</i>			
A	154	7.9	16.3	++	++	0
B	109	7.6	12.7	+++	+	0
			Highly variable			
C	98	8.1	3-27	++++	++++	++
H	41	2.4	4.4	0+	0+	0
K	52	2.0	5.1	0++	0+	0
L	154	10.5	23.2	++++	0+	0

ate 6.6 gm. per day. However, the difference in liver fat concentrations of these two groups, as shown in table 2, was not impressive. The basis for this distinction is not apparent but may lie in a refection-like process facilitated by the presence of starch and providing accessory factors which were not included in the diets. This situation is the reverse of that observed by Griffith ('41) in his studies of renal damage due to choline deficiency. It was concluded that for the purposes of this study starch was preferable to sucrose as the dietary carbohydrate.

Group C was fed a ration containing 10% lard, 10% cottonseed oil and 0.5% cholesterol, with 5% casein and starch making up most of the balance. Of twelve rats in this group,

four survived for the entire experimental period and the mean survival time of the others was 97 days. These animals grew slowly through almost the entire experiment although some lost weight during the last week or two. Four of the animals that died prematurely appeared to gain weight rapidly during the last few days but this was due to the accumulation of ascitic and pleural fluid. The livers of this group showed marked fatty change and extensive necrosis. In some livers there was nothing left but masses of ceroid within scar tissue and there were huge areas in which no liver cells remained. The mass that composed the liver was really the ceroid and other fatty material lying in bands of connective tissue. The latter mass was made up of spindle-shaped fibroblasts in the more recently destroyed areas but in the older areas consisted of pink staining bands resembling collagen. Where liver cells were seen, they were found in small islands surrounded by delicate bands of connective tissue. Most of these remaining liver cells showed considerable fatty change. On the other hand there were a few circular islands of apparently normal liver cells, which appeared to be areas of regeneration. The results of chemical determinations of fat content in this group were quite variable (table 2). Some of the most extensively necrotic livers were found to have only moderate fat concentrations, 3 to 10%, while others were extremely high, 15–25%. When groups of six rats were sacrificed 30 and 60 days after they were put on diet C, liver fat concentrations uniformly above 15% and as high as 30% were found. This must indicate that the necrotic livers of low fat content were at one time highly fatty but the fat disappeared with the death and subsequent phagocytosis of the liver cells. This process, then, is not primarily a fibrosis, but a fat induced necrosis with subsequent scar replacement. It must be recognized that were it not for the ceroid, which is apparently not an integral part of this process but accumulates only when appreciable quantities of cod liver oil are fed (Endicott, '44; Wachstein, '45), these livers would have been much smaller and a larger percentage of their total mass would have been fibrous tissue.



To ascertain the optimal protein content of the diet for the production of hepatic damage due to choline deficiency, groups D and E were fed diets similar to C but in which the casein level was raised to 7 and 10%, respectively. Of the rats in group E, 80% died within the first 12 days of the experiment and in all of these were found the grossly hemorrhagic kidneys associated with acute choline deficiency in the very young growing rat. More surprising, however, was the fact that virtually all of the animals in group D died after 5 to 7 weeks and in these animals also were found grossly hemorrhagic kidneys. Their livers, while very fatty, showed practically no necrosis or fibrosis. This was not surprising in view of the comparatively brief duration of the experiment. These animals will be described in detail in a subsequent report.

Not included in the table were two other groups which received the diet of group C supplemented with 0.35% choline chloride and 0.7% methionine, respectively. One of the ten animals in each group died prematurely but these, like the others, showed normal liver fat concentrations and no evidence of liver necrosis. Under these circumstances, it was concluded that the diet of group C, containing 5% casein, with starch as the carbohydrate and a considerable quantity of fat and cholesterol was optimal for the production of hepatic damage due to choline deficiency.

*The effects of choline deficiency without fatty  
metamorphosis of the liver*

The conditions found most favorable for the development of choline deficiency hepatic cirrhosis were also those which most promptly induced the accumulation of excessive liver fat. This is in accord with the principles stated by Connor ('37). To further test this hypothesis it was decided to study the fate of animals on a choline deficient regime so modified that no accumulation of excessive liver fat would occur. Several attempts were made to produce such conditions.

The first of these was excessive nicotinamide feeding. If 2% of nicotinamide is included in an 8% casein, high fat diet

(group F) the animals lose weight and fail to show fatty livers, even though the cause of growth failure is methionine, and consequently choline, deficiency (Handler and Dann, '42; Handler, '44). However, this phenomenon had only been studied in experiments of 14- and 28-day duration. When a longer trial was made, the animals in this group appeared to fall into two classes: those which lost weight steadily and

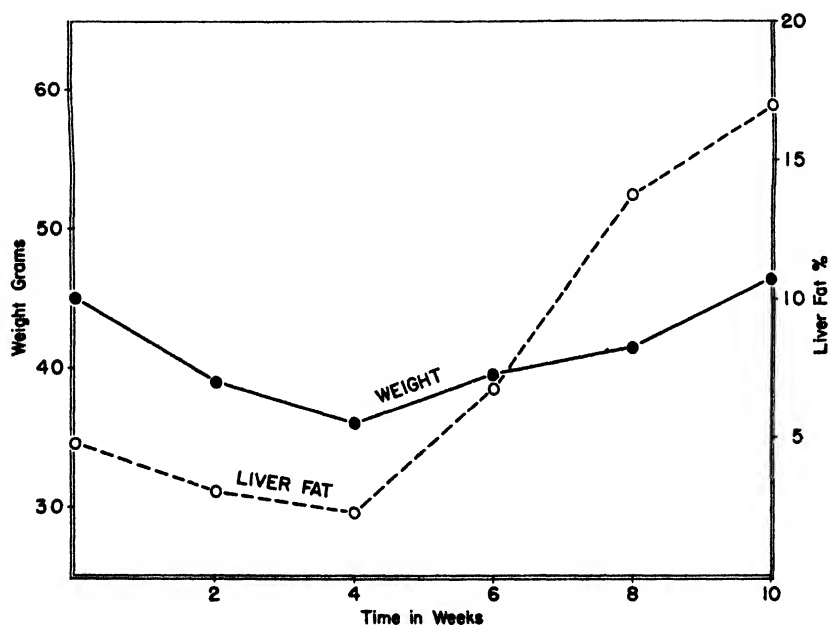


Fig. 1 Relationship between growth and liver fat concentration in excessive nicotinamide feeding.

died, presumably of methionine deficiency, and those which reached and maintained a weight plateau, after which their liver fat concentrations rose steadily. A few animals actually began to gain weight after 4 to 5 weeks and these showed a considerably elevated liver fat content. These data are summarized in figure 1, the figures representing mean values obtained with groups of five rats each sacrificed at 14-day intervals. This procedure, therefore, seemed fruitless for our purposes.

A second attempt was made, utilizing the fact that young rats on a low protein, high fat diet with lactose as the dietary carbohydrate show very little increment in liver fat content and no hemorrhagic kidneys (Artom and Fishman, '45; Handler, unpublished data). However, when a diet similar to that of group C was fed, with lactose substituted for starch, all the animals died after 14 to 22 days, a phenomenon not observed in the earlier experiments of 7-day duration. This has since been reported by Ershoff and Deuel ('44) and the mechanism is now being studied in detail and will be reported later. Consequently, this technique also was discarded.

Rats fed a diet concomitantly deficient in thiamine and choline do not show the characteristic fatty livers of choline deficiency (McHenry, '37). This is not due to any specific effects on fat metabolism, but, rather, appears to be due to the anorexia, and is but one instance of the generalization that rats which are losing weight or in negative nitrogen or caloric balance, do not develop fatty livers despite choline deficiency (Handler, '43; Handler and Bernheim, '43; Boxer and Stetten, '44). Group H consisted of twenty-four rats which were fed a diet similar to that of group C but lacking thiamine. These animals were started at an initial weight of 70 gm. and after they had lost about 20 gm. were given weekly doses of thiamine, which varied from rat to rat, such that in no instance did the weight of any rat exceed 55 gm. and most of them actually dropped below 50 gm. Only thirteen rats survived for 120 days, the others dying, apparently, of thiamine deficiency. The livers of all the rats which died prematurely were found to have unusually low concentrations of liver fat. Of the others, most had small but apparently normal livers. The maximum liver fat concentration found in the group was 7.8%. Histologically these showed little or no fatty infiltration and virtually no necrosis was seen.

Group K was composed of twelve rats which were pair fed with members of group C and fed the same ration, but whose daily food intake was limited to about 50-60% of that of the members of group C. Two of these failed to survive the ex-

perimental period. Of the remainder, four actually gained some weight over this period, albeit very slowly, and the remainder either lost weight or remained almost constant. The animals which did gain in weight were found to have somewhat elevated liver fat concentrations while the others were normal or almost so. No necrosis or fibrosis was observed in the livers of normal fat concentration, while only a slight degree of focal necrosis with minimal fibrosis was found in the livers of elevated fat content. Groups H and K, then, afford examples of situations in which rats were maintained for lengthy periods on choline deficient regimes but under circumstances in which virtually no accumulation of excessive fat occurred, nor was there any appreciable necrosis or fibrosis. This suggests that hepatic necrosis, in choline deficiency, not only occurs under those circumstances which produce an extensive fatty metamorphosis but may actually be the end result of this process.

*The effects of choline deficiency on rats fed a relatively high casein diet*

All of the diets used above, while choline deficient, were complicated by the fact that they were, of necessity, low protein diets. This was done to keep the methionine content at a minimum. Choline deficiency on relatively high protein intake has only been studied with fibrin or peanut meal rather than casein as the dietary protein. It was thought of interest to study the effects of choline deficiency in the presence of an adequate level of casein intake. Group L, therefore, was offered an 18% casein diet whose effective methionine content was reduced by the addition of nicotinamide. Thirty-six rats were used and batches of six each were killed at the end of 2, 4, 8, and 12 weeks and their livers taken for fat analysis. Two animals died unexpectedly and their livers were included with those of the remaining ten which were sacrificed after 120 days and samples taken for both chemical and histological examination. As shown in table 3, this device was successful

in that high liver fat concentrations were maintained throughout the experimental period. An attempt was also made to determine the effective methionine content of this diet. This was done by placing groups of two rats each in metabolism cages and urine collections were made for a 48-hour period and the N'-methylnicotinamide content estimated. While the first values were determined by the procedure of Huff and Perlzweig ('43), the later figures were obtained by the method of Huff, Perlzweig and Tilden ('45). Recovery experiments after giving known amounts of N'-methylnicotinamide chloride to normal rats of comparable size were also performed. When

TABLE 3

*Liver fat concentration during course of moderate nicotinamide feeding on a high casein diet.*

TIME	RAT WEIGHT	LIVER FAT	TIME	RAT WEIGHT	LIVER FAT
<i>days</i>	<i>gm.</i>	<i>% wet weight</i>	<i>days</i>	<i>gm.</i>	<i>% wet weight</i>
0	43	4.9	56	112	22.3
14	64	12.0	84	137	22.4
28	92	18.1	120	154	23.2

nicotinamide is given to rats, about one-fifth of the dose can be recovered in the urine as N'-methylnicotinamide (Huff, '45). Furthermore, when the latter compound itself is given, only about one-fifth of it can be recovered in the urine. From these considerations, it has been surmised that the four-fifths of unrecovered nicotinamide was also methylated and this compound then destroyed or further altered. Therefore, in table 4 are presented two columns representing the amount of available methionine if this assumption is correct (B) and also based directly on the urinary content of N'-methylnicotinamide (A). The methionine content of casein was assumed to be 3.5% (Block and Bolling, '45). The urinary recovery of N'-methylnicotinamide, as a percentage of the administered nicotinamide, decreased somewhat with time. It cannot be stated whether this represents decreased synthesis or increased destruction of the N'-methylnicotinamide. Through

most of the experiment, however, the effective methionine content of the diet was equivalent to a diet containing either 15% or 6% casein, depending on the validity of the assumption above. The extremely fatty livers make the latter values seem more nearly correct.

TABLE 4

*The amount of effective methionine in a diet containing excess nicotinamide. All values are expressed in terms of one rat for 1 day.*

TIME	FOOD INTAKE	METHIONINE IN FOOD	NICOTIN-AMIDE INTAKE	METHYL-NICOTIN-AMIDE EXCRETED	EFFECTIVE METHIONINE		METHYL-NICOTIN-AMIDE INTAKE
					A	B	
day	gm.	mg.	mg.	mg.	mg.	mg.	mg.
28	8.3	58	100	7.8	50	16	
118	11.5	73	138	8.6	64	26	
1				38			220

TABLE 5

*Unavailability of N'-methylnicotinamide for choline and methionine synthesis. All values represent means for groups of 6 rats, for an experimental period of 7 days.*

SUPPLEMENT	WEIGHT GAIN		FOOD INTAKE	LIVER FATTY ACIDS
	gm. per day	gm. per day		% wet weight
None	2.8	7.8		21.6
Choline	2.8	7.7		5.1
Methionine	3.1	8.2		4.6
N'-methylnicotinamide	2.5	6.9		18.0
N'-methylnicotinamide + homocystine	2.3	7.2		18.7

The calculations and assumptions above predicated that the methyl group of N'-methylnicotinamide was not available for the resynthesis of methionine or choline. This has been indicated by the work of Handler and Dann ('42) but more concrete evidence is presented here. Group 1 was fed diet E, similar to that of group C but containing 10% of casein. Group 2 received the same diet with a supplement of 0.5% choline; group 3 received 0.7% methionine; group 4 received 1% of N'-methylnicotinamide chloride, while group 5 received 1% N'-methylnicotinamide plus 0.7% dl-homocystine. The data are summarized in table 5. It will be seen that while choline

and methionine completely prevented the excess liver fat accumulation, N'-methylnicotinamide alone or in combination with homocystine failed to exert lipotropic activity.

Of the twelve rats in group L sacrificed at the end of 120 days, five showed only minimally fatty livers with no necrosis or fibrosis. The remaining seven livers were extremely fatty yet showed only very slight necrosis with minimal fibrosis. The necrosis, such as it was, appeared to commence around the central vein. The changes were manifest as hyalinization and poor staining of the liver cells with subsequent loss of liver parenchyma and accumulation of ceroid within a slight amount of scar tissue. This group then, stands in contrast to groups A and C. Despite the persistent fatty degeneration only a minimal amount of necrosis was evident when the animals were sacrificed. It seems likely that the necrosis might have been more extensive if the experiment had been prolonged, but the results would not then have been comparable with groups A and C. Thus, while the accumulation of fat in the livers of animals on low protein diets, in analogy with human alcoholic or Laennec cirrhosis, appears to lead to necrosis with subsequent fibrosis, a reasonably high protein intake seems to afford a degree of protection against the deleterious effects of fat accumulation noted on low protein diets.

#### DISCUSSION

In agreement with all previous reports it was found that high fat, low protein diets provide optimal conditions for the production of hepatic necrosis and fibrosis. These conditions also proved optimal for the accumulation of liver fat. When these conditions were altered either by thiamine deprivation or simply restricting food intake the fatty infiltration was minimal and virtually no necrosis was observed. This suggests that the liver necrosis of choline deficiency may be the obligatory consequence of massive fatty infiltration of the parenchymatous liver cells and that only the lipotropic activity of choline is involved in maintaining the integrity of the liver.

This is in accord with Connor's ('37) concept of the pathogenesis of alcoholic cirrhosis in man.

The data reported herein do not afford an explanation of the mechanism by which the accumulation of fat may result in the death of parenchymatous liver cells. It is certain that such cells are highly organized systems and that the integrity of this organization is necessary for the proper functioning of the cell. The fat within liver cells is either absorbed from the surrounding medium or is synthesized *de novo* from carbohydrate and protein precursors. However, to leave the liver as phospholipid, choline is necessary and, in choline deficiency this fat accumulates, appearing first as small discreet droplets which later coalesce. As more fat collects the cytoplasm and nucleus can be found together in one corner of the cell whose bulk is now largely the fat droplet. However, as still more fat appears, it seems not unreasonable to believe that the physical organization of the cytoplasm and nucleus is disrupted with consequent loss of function and death. With the disintegration of the cell the fat is removed by phagocytosis and scar tissue then develops in its place.

On the other hand the behavior of the rats of the high casein, nicotinamide containing diets has demonstrated that liver cells may survive despite prolonged fatty infiltration. These data are in contrast with those obtained after feeding diets containing relatively large amounts of fibrin or peanut meal. The fat concentrations in the livers of group L were as great as those seen during the development of liver necrosis and fibrosis under our optimal conditions. Yet virtually no necrosis was found. No evidence is available concerning the fate of the methionine moiety after transmethylation and N'-methylnicotinamide synthesis although it has been assumed that homocysteine is formed and the latter converted to cysteine and cystine. It remains possible that the apparent protection afforded the liver by this high casein diet resides in this methionine derivative, but this protective capacity is not based upon lipotropic activity. It is, however, consistent with Gyorgy's ('44) findings that choline plus cystine is more



effective than choline alone in the prevention of necrosis and cirrhosis, and perhaps this situation is similar to the frequent post-mortem finding of highly fatty livers with little or no necrosis. Further study of the possible role of other amino acids and such factors as streptogenin is warranted.

Recently Himsworth and Glynn ('44 a, b) have distinguished between an acute, massive, hepatic necrosis, analogous to acute yellow atrophy in man, which they state to be due, specifically, to methionine deficiency (but despite rather than because of its lipotropic activity) and a progressive, diffuse hepatic fibrosis which is not accompanied by necrosis but thought to be due to fatty infiltration caused by lack of sufficient dietary lipotropic factors (methionine, choline, etc.) and preventable by dried yeast. Comparison of their data with ours is difficult because of the many differences in technique and the diets employed. Since the lesions we have studied were actually post-necrotic scars they would consider our results to be the combination of their two supposedly, independent, disease processes. It is difficult indeed to understand the development of scar tissue in a liver which is composed of living, albeit fatty cells. We have observed no process similar to their massive necrosis and believe that this is because of the inclusion of sufficient cystine in all of our diets. While they found that methionine was preventive but cystine was not, it must be noted that while they used supplements of 20 mg. per day of methionine, they tried cystine only at the level of 2.2 mg. per day. In contrast our diets provided about 30 mg. of cystine per day which should have been ample to prevent the specific necrosis of cystine deficiency (Weichselbaum, '35; Daft et al., '41) which is apparently similar to these authors' acute, massive necrosis. The protective action of methionine may well have been due to its conversion to cystine. Since the livers of our choline deficient rats were definitely necrotic despite the presence of adequate dietary cystine, and since choline supplements to such a diet completely prevented the appearance of any form of demonstrable hepatic damage, we must conclude that the hepatic fibrosis of choline deficiency is a form of postnecrotic scarring.

## SUMMARY

1. Diets high in fat containing 4-5% casein with starch as the carbohydrate and supplemented with cystine and cholesterol proved most suitable for the production of choline deficiency liver necrosis and fibrosis in albino rats.

2. Thiamine deficiency or restricted food consumption prevented the accumulation of excessive liver fat and also liver necrosis.

3. N'-methylnicotinamide was found to exert no lipotropic activity alone or in combination with homocystine.

4. Inclusion of excess nicotinamide in an 18% casein diet produced extensive fatty infiltration but only minimal necrosis of the liver.

5. It is suggested that the hepatic necrosis and fibrosis of choline deficiency may be the result of chronic fatty infiltration. The ingestion of an adequate quantity of good protein protects the liver from the deleterious effects of chronic fatty infiltration but this protective capacity is not based upon lipotropic activity alone.

## ACKNOWLEDGMENTS

This work was supported by grants from the Josiah Macy, Jr., Foundation, the Duke University Research Council, and a Charles Mayer Fellowship of the New York Academy of Medicine. Our thanks are due to Dr. Roger D. Baker who made the histological examination of some of the material reported herein and to Merck and Company for a supply of crystalline vitamins.

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## PLATE 1

### EXPLANATION OF FIGURES

All sections were stained with hematoxylin and eosin.

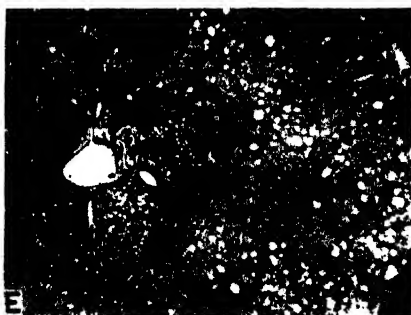
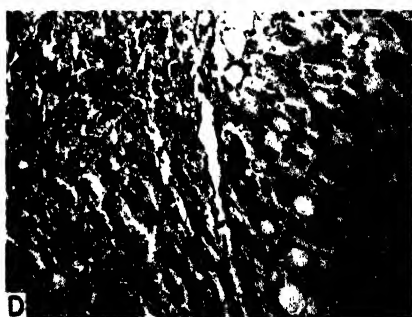
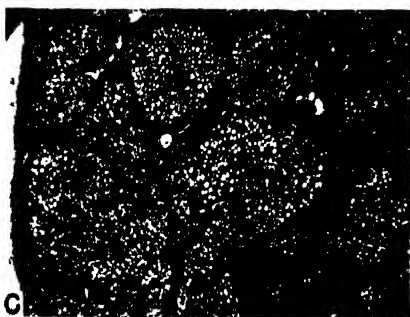
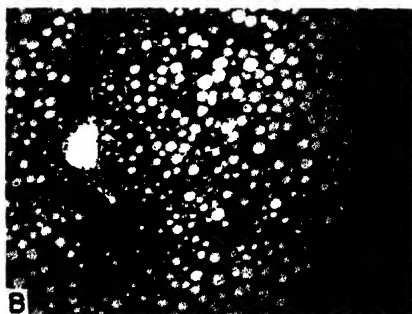
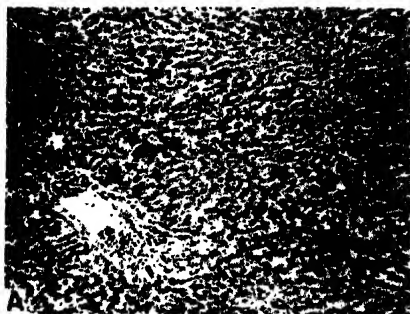
A. Normal liver; group K; restricted intake of optimal cirrhosis producing diet.  $\times 137$ .

B. Liver of typical rat in group L; nicotinamide added to high casein diet; note lack of necrosis despite extensive fatty change, especially in central portion of lobule.  $\times 137$ .

C. Liver of rat in group C; optimal diet for producing cirrhosis. This liver still shows a marked fatty change with but a moderate degree of cirrhosis. The liver substance is arranged in irregular islands demarcated by connective tissue in which the ceroid is visible as non-staining refractile white dots.  $\times 60$ .

D. Another liver from group C. This area is at the periphery of an island of liver tissue and shows the numerous globules of ceroid lying in a meshwork of newly formed connective tissue and capillaries.  $\times 295$ .

E. Another liver from group C. This is an extreme degree of liver necrosis. In the area shown practically all the liver cells have died so that the mass consists of small globules of ceroid lying in connective tissue. Some globules of fat still remain.  $\times 60$ .





# VITAMIN C CONTENT OF MARKET MILK, EVAPORATED MILK, AND POWDERED WHOLE MILK

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## TWO FIGURES

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The vitamin C content of cows' milk has been studied by a large number of investigators. Booher, Hartzler and Hewston ('42) and Mendive and Repetto ('40) have made compilations of values found in the literature. Much of the data concerning the vitamin C content of milk has been obtained with the object of studying factors which influence the amount originally present in the milk and its stability under various conditions. Comparatively little data have been published concerning the actual amount of vitamin C in milk and milk products produced commercially and sold to consumers.

An extensive survey of the ascorbic acid content of commercial pasteurized bottled milk was conducted by Guthrie, Hand, and Sharp ('38), who found an average of 2.9 mg. reduced ascorbic acid per liter as the result of analyzing 1502 samples from dairy plants in the State of New York 24 hours after delivery. Hawley ('38) found an average of 9 mg. reduced ascorbic acid per liter in 100 samples of commercial pasteurized milk taken from milk wagons in Rochester, New York. Samples from five dairies in Madison, Wisconsin, were analyzed by Woessner, Elvehjem and Schuette ('39) and found to contain an average of 8.9 mg. reduced ascorbic acid and 12.6 mg. total vitamin C per liter. Among those studies



made on milk sold in foreign cities is that of Mendive and Repetto ('40), who found 11.3 mg. to 22 mg. total vitamin C per liter in pasteurized milk sold in Buenos Aires, approximately half being in the form of dehydroascorbic acid; Palladina and Anashkina ('37) found an average of 16.5 mg. vitamin C per liter in milk sold in Leningrad.

The importance of dehydroascorbic acid as a source of vitamin C has not been realized, nor suitable methods for its analysis devised until recent years. The necessity for determining dehydroascorbic acid in addition to reduced ascorbic acid has been shown by Hand ('43), who states that determinations of reduced ascorbic acid alone may be in error as a measure of total vitamin C by amounts ranging from 0 to 6 mg. per liter.

Sharp, Guthrie and Hand ('38, '40, '41, '42) have made comprehensive studies of the stability of vitamin C in milk. A total of 1187 samples of fresh milk were analyzed in which the reduced ascorbic acid content was found to range from 7 to 41 mg. per liter with an average value of 22.2 mg. Pasteurization was found to accelerate the destruction of ascorbic acid only in the presence of dissolved oxygen and copper. Season and feed of the cow exerted little effect upon the ascorbic acid content of the fresh milk. The same authors studied losses of vitamin C in milk in commercial milk plants from the time of its delivery to the country milk plant to bottling in New York City. Milk in the patrons' cans at the country shipping plant averaged 18.9 mg. per liter, weigh tanks 18.5 mg., pump reservoir 18.4 mg., tank truck 18.3 mg., and tank cars 17.7 mg. In one plant in New York City, using holder pasteurization, the milk entering the weigh tank averaged 15.7 mg., before pasteurizer 15.7 mg., after pasteurizer 11.1 mg., after cooler 9.1 mg. and in the bottle 9.1 mg. A similar study as the milk passed through a New York City plant using short-hold-high-temperature pasteurization showed that milk entering the plant averaged 15.7 mg., after pasteurization 15.0 mg., and in the bottle 14.3 mg. The causes for losses in vitamin C are shown to be the result of copper contamination

and presence of dissolved oxygen, during pasteurization and especially on holding after pasteurization.

The vitamin C content of evaporated milk has been stated by various investigators (Doan and Josephson, '43; Henry et al., '38; Meulemans and de Haas, '38; Schlemmer et al., '32; Taniguchi et al., '37; Tomoi and Tomita, '37; and Woessner et al., '40) to range from practically none to more than that found in fresh milk. It has been pointed out by Woessner, Elvehjem and Schuette ('40) that in most of the earlier work methods were employed in which there was no evidence presented as to their specificity for vitamin C. This no doubt explains for the most part the wide variation in values found in the literature. More recent values obtained with methods designed to correct for interfering substances are those of Woessner, Elvehjem and Schuette ('40) who found 1.9 mg. to 11.2 mg. per liter of reconstituted evaporated milk purchased from stores, while an average of 4.1 mg. per liter was obtained by Doan and Josephson ('43).

Although there are a number of references in the literature (Barcroft, '43; Henry et al., '39; Hochberg et al., '43; Jung, '40; Meulenians and de Haas, '38; Renner, '36; Schlemmer et al., '32; Tomoi and Tomita, '37; and Woessner et al., '40), almost no comprehensive data are available concerning the vitamin C content of powdered whole milk. Analysis has generally been carried out on only a few samples and values vary greatly among different investigators. The same criticism as to lack of evidence for the specificity of the methods employed for the analysis of evaporated milk can be applied to most of the data for vitamin C in powdered milk.

#### METHODS

Reduced ascorbic acid in market milk was analyzed by indophenol titration according to the method of Sharp ('38). Dehydroascorbic in market milk was determined after conversion to reduced ascorbic acid with a suspension of *Escherichia coli* as described by Gunsalus and Hand ('41).

Evaporated milk contains large amounts of reducing substances which interfere with the indophenol titration method. This is also true to a lesser degree with powdered whole milk. To obtain true values for vitamin C the authors' selective oxidation-reduction method (Stewart and Sharp, '45) was used. In this method the enzyme (or enzymes) in cucumber juice is used to destroy interfering substances, while also converting reduced ascorbic acid to dehydroascorbic acid. By subsequently adding a suspension of *Escherichia coli* or *Staphylococcus albus* the ascorbic acid is recovered in the reduced form and any dehydroascorbic acid originally present is also converted to reduced ascorbic acid, which can be titrated with indophenol dye.

#### EXPERIMENTAL AND RESULTS

##### *Market milk*

Studies were made to determine the total vitamin C content of milk under conditions representative of those in the consumer's home. For this purpose, samples of commercial pasteurized milk were brought to the laboratory for analysis directly from homes of actual subscribers to home delivery milk and from retail stores. Since much of the milk was delivered during the afternoons, the samples were placed in a refrigerator at 10°C. and analyzed the next morning following date of delivery. This time was taken as the most probable for use in the home. A total of 364 samples were analyzed — 237 quart cartons sold in retail stores and 127 quart glass bottles sold on home delivery routes.

This survey was made in the San Francisco — Oakland — Berkeley Bay Region from May 20 to September 21, 1943. Ten major delivery plants were represented, supplying approximately 80% of the milk sold in this region.

Table 1 gives the average values for each of the 10 plants. The variation in average total vitamin C among the plants was 2.9 mg. to 10.0 mg. per liter. Most of this variation was in the reduced ascorbic acid content, which ranged from 0.8 mg. to

7.9 mg. per liter, dehydroascorbic acid varying only from 1.7 mg. to 3.5 mg. per liter.

The reduced ascorbic acid content of all 364 samples averaged 3.4 mg. per liter, dehydroascorbic acid 2.4 mg. per liter, and the sum of the two, or total vitamin C, 5.8 mg. per liter. Individual samples ranged from 0 to 14.3 mg. reduced ascorbic acid, 0 to 8.2 mg. dehydroascorbic acid and 0 to 15.2 mg. total vitamin C per liter.

TABLE 1

*Vitamin C content of commercial pasteurized milk consumer samples of day following date of delivery.*

PLANT NO.	NO. OF SAMPLES	TYPE OF CONTAINER (1 QUART)	PLANT AVERAGE VALUES		
			Reduced ascorbic acid	Dehydro-ascorbic acid	Total vitamin C
			mg./liter	mg./liter	mg./liter
1	29	carton	7.9	2.1	10.0
2	26	carton	4.2	2.1	6.3
3	24	carton	6.4	1.9	8.3
4	27	carton	4.8	2.8	7.6
5	24	carton	4.1	3.3	7.4
6	27	carton	1.2	2.7	3.9
7	23	carton	2.1	1.7	3.8
8	28	carton	1.0	2.7	3.7
9	29	carton	1.5	2.2	3.7
10	36	bottle	1.0	1.9	2.9
1	40	bottle	5.0	2.6	7.6
2	29	bottle	3.8	2.3	6.1
8	22	bottle	0.8	3.5	4.3
Total	364	Ave.	3.4	2.4	5.8

There is a very marked tendency for samples of milk with low reduced ascorbic acid content to contain relatively large amounts of dehydroascorbic acid. Also samples low in dehydroascorbic acid tend to be high in reduced ascorbic acid. This is shown in figure 1, where the largest proportion of samples fall in the lower ranges relative to reduced ascorbic acid and dehydroascorbic acid, but total vitamin C, the sum of reduced ascorbic acid and dehydroascorbic acid, maintains

a fairly constant distribution up to and including the 8 to 10 mg. interval.

In order to further simulate conditions existing in the homes of milk consumers, a total of 110 samples — 44 bottles and 66 cartons — were held another day in a household type of electric refrigerator at approximately 10°C. and again analyzed for vitamin C. A loss of 30% total vitamin C resulted from this additional day's holding.

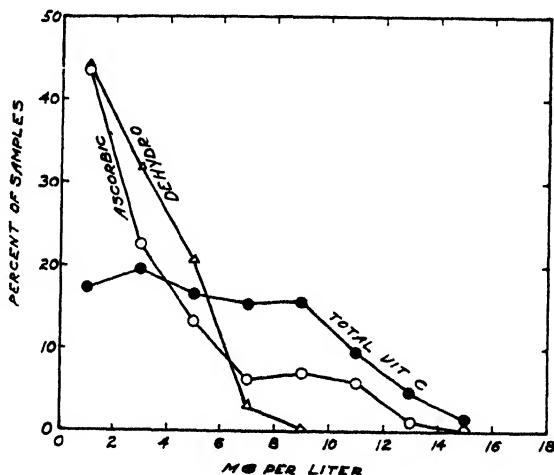


Fig. 1 Vitamin C distribution curves for 364 samples of market milk from consumers' homes and retail stores.

### *Evaporated milk*

Samples of evaporated milk were purchased in retail stores located in San Francisco and Oakland during the months of April, 1943, January, 1944 and May, 1945. Twelve major brands were represented in a total of twenty-five samples analyzed.

Table 2 shows that evaporated milk contains only small amounts of reduced ascorbic acid and little or no dehydroascorbic acid. Reduced ascorbic acid averaged 1.9 mg., dehydroascorbic acid 0.1 mg., and total vitamin C 2.0 mg. per liter of reconstituted evaporated milk. For individual samples the range in

TABLE 2

*Vitamin C content of commercial evaporated milk, reconstituted by diluting with equal part of water.*

BRAND	NO. OF SAMPLES	ASCORBIC ACID	DEHYDRO- ASCORBIC ACID	TOTAL VITAMIN C
			<i>mg./liter</i>	
1	3	2.2	0.0	2.2
2	1	0.3	0.0	0.3
3	3	2.9	0.6	3.5
4	3	0.7	0.3	1.0
5	2	0.8	0.0	0.8
6	3	2.6	0.2	2.8
7	2	0.4	0.0	0.4
8	1	4.3	0.0	4.3
9	3	0.5	0.2	0.7
10	1	3.8	0.0	3.8
11	2	0.5	0.1	0.6
12	1	3.7	0.3	4.0
Total	25	Ave. 1.9	0.1	2.0

reduced ascorbic acid was 0.0 mg. to 6.6 mg., dehydroascorbic acid 0.0 to 1.3 mg. and total vitamin C 0.0 to 6.9 mg. per liter.

### *Powdered whole milk*

A total of 2890 samples of spray process powdered whole milk were analyzed for total vitamin C. The samples were not over a few days old and were obtained chiefly from three plants, although over 100 samples were obtained from a number of other plants over the country. Also a total of 323 samples were analyzed after storage at room temperature in one pound, air packed tins for 3 months, 651 samples after 6 months' storage, and 400 samples after 12 months' storage. Each sample represents powdered whole milk dried from a separate batch of milk.

The vitamin C in powdered whole milk is largely in the form of reduced ascorbic acid, little or no dehydroascorbic acid being present. For this reason, and for convenience, the data have been expressed in terms of total vitamin C without reference to relative proportions of the two forms. Values are

given in terms of milligrams total vitamin C per 125 gm. powdered whole milk, or the amount required to make one liter of reconstituted whole milk.

The mean average of the 2890 samples of fresh powdered whole milk for the period December, 1942, through May, 1945, was 12.5 mg. vitamin C per 125 gm. The value of 12.3 mg. per 125 gm. was obtained by averaging the data by months. This is shown by the data given in table 3.

TABLE 3

*Vitamin C content of fresh spray process powdered whole milk.*

MONTHS	NO. OF SAMPLES	MEAN AVE. MG./125 GM
Dec., Jan., Feb., 1942-1943	128	11.8
Mar., Apr., May, 1943	96	10.9
June, July, Aug., 1943	237	11.4
Sept., Oct., Nov., 1943	107	12.9
Dec., Jan., Feb., 1943-1944	247	12.0
Mar., Apr., May, 1944	211	12.3
June, July, Aug., 1944	144	13.2
Sept., Oct., Nov., 1944	110	13.2
Dec., Jan., Feb., 1944-1945	466	12.8
Mar., Apr., May, 1945	1144	12.8
Total	2890	Ave. 12.3

The combined data are presented in the form of a distribution curve by figure 2. The median value was 12.6 mg. vitamin C per 125 gm. powdered whole milk. Only 4 samples contained less than 5 mg. whereas 2726 (94.3%) contained over 10 mg. per 125 gm.

To study the effect of storage on the vitamin C content of powdered whole milk, samples were analyzed fresh and were air packed in either duplicate or triplicate in one pound tin cans and stored at room temperature (22°C.) for periods up to 12 months. Although in every case the same sample was analyzed fresh and also at the end of a certain storage period, in many instances the same sample was not analyzed at each of the three storage periods. The data are presented in table 4.

After storage for 3 months, the average loss in vitamin C was only 1.4 mg., after 6 months, the loss was 1.8 mg., and after 12 months the loss was 2.5 mg. Even smaller losses in vitamin C have been found in the case of samples which were nitrogen packed.

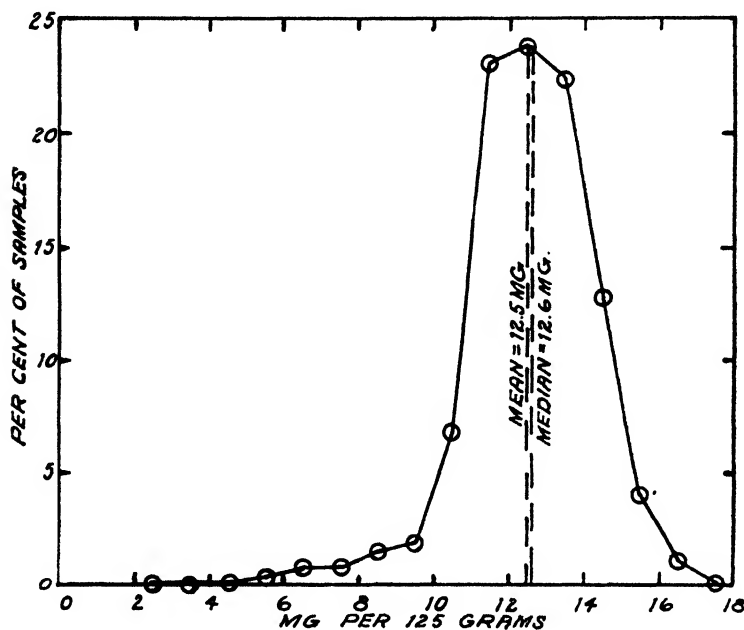


Fig. 2 Vitamin C distribution curve for 2890 samples of fresh spray-process powdered whole milk.

TABLE 4

*Effect of storage at room temperature (22°C.), air packed, on the vitamin C content of spray process powdered whole milk.*

PERIOD OF MANUFACTURE	NO. OF SAMPLES	VITAMIN C — MG./125 GM.			
		Fresh	3 Mos.	6 Mos.	12 Mos.
Dec., 1942–Feb., 1944	323	12.4	11.0		
Dec., 1942–Sept., 1944	651	12.4		10.6	
Dec., 1942–March, 1944	400	12.3			9.8



*Raw milk received at plants*

In order to compare values for raw milk with those obtained for market milk, evaporated milk and powdered whole milk, reduced ascorbic acid was determined in a number of samples taken from milk delivered to milk plants by various patrons. Dehydroascorbic acid was not determined, since it is so unstable to pasteurization that it cannot be preserved under even the most ideal processing conditions. A total of 1050 samples were analyzed during five separate periods upon receipt at various plants. The average reduced ascorbic acid content for the five periods ranged from 16.1 mg. to 18.6 mg. with an over-all average of 17.1 mg. per liter. This is slightly less than the value of 18.9 mg. found previously by Sharp, Guthrie and Hand ('40).

## DISCUSSION

Market milk from consumers' homes and from retail stores averaged 3.4 mg. reduced ascorbic acid, 2.4 mg. dehydroascorbic acid and 5.8 mg. total vitamin C per liter with 30% loss on holding another day. The value for reduced ascorbic acid is in good agreement with that of 2.9 mg. per liter found by Guthrie, Hand and Sharp ('38).

Evaporated milk contained 1.9 mg. reduced ascorbic acid and 0.1 mg. dehydroascorbic acid per liter reconstituted. The value found for vitamin C in evaporated milk is lower than most of the results found in the literature. Our experience with the methods previously used has shown them to give slightly high results in some instances since interfering substances were not entirely eliminated.

The mean value for fresh spray process powdered whole milk was 12.5 total vitamin C per liter reconstituted (125 gm. powder). The median value for the same samples was 12.6 mg. per liter. No extensive data could be found in the literature with which to compare these values. Woessner, Elvehjem, and Schuette ('40) found 10.6 mg. total vitamin C in one sample of spray powdered whole milk, of which 9.4 mg. was in the form of reduced acid. Henry, Houston, Kon, and Os-

borne ('39) found 15.5 mg. total vitamin C per liter reconstituted, which is probably too high since it includes 4.6 mg. dehydroascorbic acid obtained by a method tending to give high results. Other values reported in the literature, mostly on a few isolated samples are 16.9 mg. (Tomoi and Tomita, '37), 48 mg. (Jung, '40), 8 to 16 mg. (Meulemans and de Haas, '38), 2.6 mg. (Hochberg et al., '43) and 2.9 to 10.0 mg. (Renner, '36).

The fact that fresh powdered whole milk averaged 12.5 mg. per liter vitamin C when reconstituted as compared with 5.8 mg. for market milk and 2.0 mg. for evaporated milk, shows that the powdered whole milk is capable of contributing an appreciable amount of vitamin C to the diet. The stability of vitamin C in powdered whole milk, even on long storage makes this product a good carrier of the vitamin.

#### SUMMARY

1. A comprehensive survey has been made to determine the vitamin C content of dairy products produced and sold commercially.

2. Pasteurized milk from consumers' homes and retail stores in a metropolitan area averaged 5.8 mg. and reconstituted evaporated milk from retail stores averaged 2.0 mg. total vitamin C per liter.

3. Fresh powdered whole milk averaged 12.5 mg. total vitamin C per liter reconstituted. Samples stored at room temperature, air packed, retained 88.7, 85.5 and 79.8% of their vitamin C content after 3, 6 and 12 months, respectively.

4. Raw milk entering the manufacturing plants averaged 17.1 mg. of reduced ascorbic acid per liter.

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# STUDIES ON THE NUTRITIVE VALUE OF FISH PROTEINS

## I. EVALUATION BY THE RAT GROWTH METHOD AND BY THE CANNON METHOD

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### TWO FIGURES

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The normal supply of animal protein has been insufficient to meet the demands for the ever increasing requirements for proteins of high nutritional value. Therefore several attempts have been made to supply this deficiency with proteins of vegetable origin. In general, however, such proteins are usually considered to have a biological value lower than those of animal origin.

The fish supply offers a large reserve of available protein which according to the experiments of Holmes ('18) with human subjects has a high digestibility. These experiments were made with butterfish, Boston mackerel, grayfish and salmon.

Unfortunately, only a relatively small proportion of the total available fish catch is at present used for human consumption in the form of fresh, dried or canned products. The balance which is transformed into fish meal or other by-products is lost for direct human nutrition.

In order to provide a method whereby it would be possible to save the proteins present in the waste products, the pro-

teins were isolated from the easily perishable components and from the factors which make such by-products unpalatable. The present experiments were carried out in order to evaluate the biological activity of the proteins isolated commercially from the edible fish by-products.

The proteins of the three types of fishes most available commercially in Southern California waters, namely mackerel (*Pneumatophorus jap. diego*), sardine (*Sardina coerulea*), and tuna (*Thunnus Thynnus* and *Sarda Chiliensis* are the principal species), were tested for their biological value in growth experiments. In the case of mackerel protein, tests were also made using the Cannon procedure ('44). Further experiments on the nutritive value of mackerel protein are afforded by bioassays for vitamin A where this protein has been substituted for casein (Deuel, Hrubetz, Johnston, Rollman and Geiger, '45).

#### METHODS

The fish proteins used were prepared from edible fish muscle by isoelectric precipitation followed by purification by means of organic solvent extraction. The mackerel and sardine proteins were uniform white powders of high purity while the tuna preparation had a brown color and from the analyses appeared to be less pure. All of the fish proteins were without flavor. The casein used in control experiments was vitamin-test casein obtained from General Biochemicals, Inc. An analysis on a technical grade of casein is also included for comparison. The average composition of these proteins is given in table 1.

The amino acid composition of various types of fish has been reported earlier by numerous investigators and these data show the presence of all essential amino acids in desirable concentrations (Clark and Clough, '26; Bottinger and Baldwin, '40; Beach, Munks and Robinson, '43; Block and Bolling, '45). Some of these data were confirmed in our laboratory. The total amino acid analyses will be reported in a later communication.

*Growth experiments*

The growth experiments were performed on Sprague-Dawley rats. They were started at 21 days of age on the diets listed in table 2.

In each of the five series of tests, twelve rats were used. The rats in each series were equally divided into two groups each consisting of three male and three female rats. One group in each series received the fish protein diet under investiga-

TABLE 1

*A comparative analysis of casein and purified fish proteins.*

COMPONENT	CASEIN		FISH PROTEINS		
	Commercial	Vitamin-test	Mackerel	Sardine	Tuna
	%	%	%	%	%
Total N (Kjeldahl)	13.75	14.30	14.31	14.28	13.05
Protein ( $N \times 6.25$ )	86.0	89.0	89.5	89.3	81.6
Water	7.0 (6.8)	4.1	7.0	6.2	6.6
Ash	2.2 (1.5-3.0)	1.9	0.8 <sup>1</sup> (0.5-1.0)	0.8 <sup>1</sup> (0.5-1.0)	0.8 <sup>1</sup> (0.5-1.0)
Fat (Ether-extractable)	1.5 (1.0-2.0)	0.3	0.5 <sup>1</sup>	0.5 <sup>1</sup>	0.5 <sup>1</sup>
Undetermined	3.3	4.3	2.2	3.2	10.5

<sup>1</sup> Approximate values.

tion while the second control group received the same level of casein. Litter mates were distributed equally between the two groups. Animals were fed ad libitum and in two groups the food consumption was determined.

*Methods used in hypoproteinemia experiments*

In general the procedures were similar to those outlined by Cannon et al. ('44). Large adult male rats from our stock colony weighing approximately 300 gm. at the start were



placed on the low protein diet employed by Cannon and continued on it for 95 days. They were then divided into three groups so that the average per cent of the original weight lost was identical in the three groups (43.8, 42.7 and 42.2%). Group I continued on the basal diet. In the diet for group II, 9% of the starch was replaced with casein and in group III 9% of the starch was replaced with the mackerel protein. At

TABLE 2  
*Composition of experimental diets.*

DIETARY COMPONENTS	SERIES 1		SERIES 2		SERIES 3		SERIES 4		SERIES 5	
	Diet A	Diet B	Diet C	Diet D	Diet C	Diet E	Diet F	Diet G	Diet H	Diet I
Casein	9		12		12		15		21	
Mackerel protein				12						21
Sardine protein								15		
Tuna protein		9				12				
Sucrose	74	74	71	71	71	71	68	68	62	62
Rice bran concentrate <sup>1</sup>	8	8	8	8	8	8	8	8	8	8
Cottonseed oil	4	4	4	4	4	4	4	4	4	4
Salt mixture <sup>2</sup>	4	4	4	4	4	4	4	4	4	4
Fish-liver oil <sup>3</sup>	1	1	1	1	1	1	1	1	1	1

To each 100 gm. of the different diets the following were added: riboflavin 1.5 mg., calcium pantothenate 3.0 mg., and chlorine chloride 50 mg.

<sup>1</sup> Supplied by the National Oil Products Co.

<sup>2</sup> U.S.P. XII no. 2.

<sup>3</sup> Contains 2000 I.U. vitamin A and 400 I.U. vitamin D per gram.

the end of 7 or 8 days, the rats were anesthetized with amytal, and a sample of approximately 0.1 ml. of blood was obtained from the tail. Plasma volume was then determined using Evans blue by the method as applied by Hechter ('45) using an interval for removal of the blood 5 minutes after the injection of the dye. Plasma protein was determined by the method of Mehl ('45) using a Klett-Summerson photoelectric colorimeter with a 540 filter and a conversion factor of 1400. Hemoglobin was estimated by the usual acid-hematin method on the Klett-Summerson colorimeter with a 420 filter.

## RESULTS

The comparative results of the growth experiments where casein or fish protein was used are indicated in figures 1 and 2.

It is evident from these figures that in all cases better growth was obtained on the diets containing the fish proteins than on the diets containing the corresponding level of casein. It is especially striking that a greater growth rate should occur in the rat receiving the mackerel protein diet than in the casein rats when the proteins were fed at a level of 21%. It is

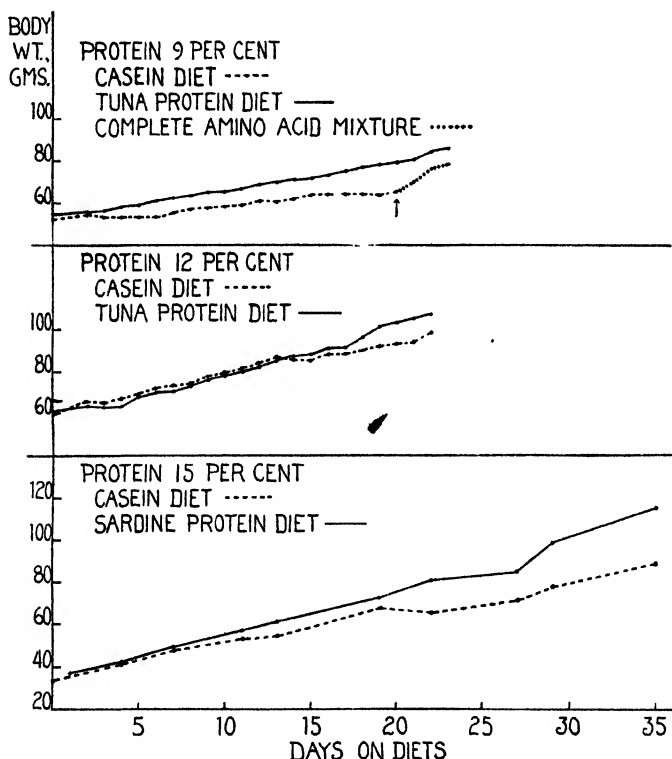


Fig. 1 The body weight of rats at various periods following weaning on casein or fish protein diets fed ad libitum. Tuna protein was used at 9% (diet B) in the experiments in the top graph, and at 12% (diet E) in those of the middle graph. Sardine protein (diet G) at 15% was the fish protein used in the lowest graph.

usually regarded that casein when fed at 18% affords an optimum growth.

The higher nutritive value of the tuna protein is suggested also by the fact that the total food consumed to give 1 gm. weight increase is much lower than for casein. In the rats receiving 9% of the tuna protein in the diet, an average of 157.6 gm. of food was consumed over the 24-day period and the average weight increase was 30.5 gm. The average ratio of food consumed to gain in weight was 5.2 (6.11, 5.88, 5.29, 5.14, 4.87, 4.35). In contrast to these values an average of 114.9

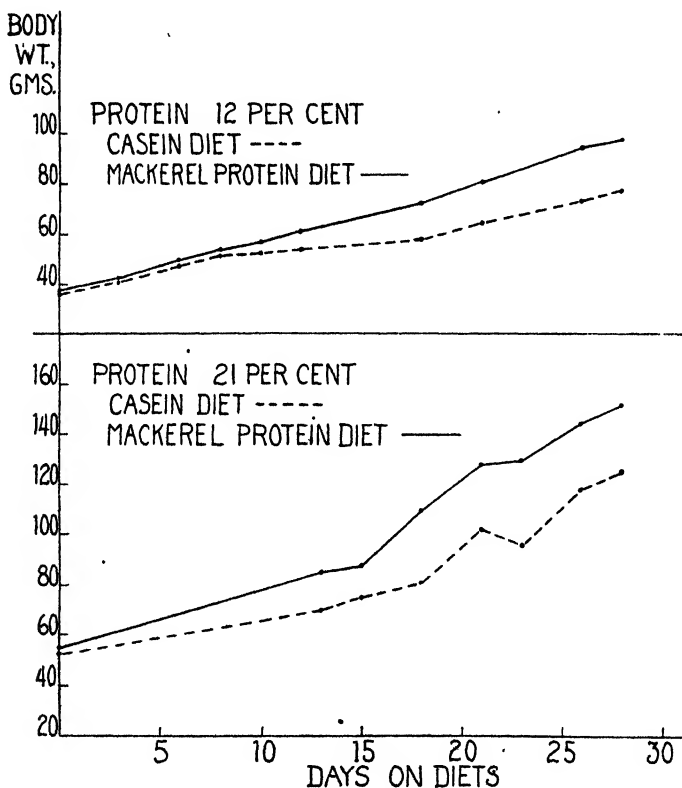


Fig. 2 The body weights of rats at various periods following weaning on casein or mackerel protein diets fed ad libitum. Diets C and D were used in the experiments given in the upper graph; diets H and I were used in the tests reported in the lower graph.

gm. of food were consumed in the control casein tests and the average gain in weight over the whole period was 8.0 gm. The mean ratio of food consumed to gain in weight was 14.3 (109.4, 28.1, 15.8, 11.8, 8.74, 8.20). It might be expected that at a higher protein level, the difference in the ratio would be less pronounced. In the experiments with 12% of protein in the food, the average ratio for the animals fed mackerel protein was 4.0 (4.11, 4.0, 4.0, 4.0, 3.97, 3.69) while that of the casein-fed rats was 4.8 (5.5, 5.1, 4.8, 4.4, 4.4).

That the differences in growth in series 1 are significant is indicated by the fact that the maximum weight of any casein rat in all determinations after the tenth day is less than the average for the fish protein group for the corresponding period.

The data on the experiments on hypoproteinemic rats are summarized in table 3 which follows.

The weight increase after the casein diet was 20.2 gm. over what would have been expected had the rats continued on the basal diet, while the rise in weight of the rats receiving the mackerel protein on a similar basis amounted to 28.8 gm. The total protein eaten during the supplement period was highest with the fish proteins rats (9.71 gm.) and slightly lower with the animals receiving the casein diet (8.14 gm.) as compared with a protein intake of 1.24 gm. for the rats consuming the basal diet.

Hemoglobin showed a rise from the subnormal value in the control rats of 8.37% to 9.83 in the casein group and 10.42 in the mackerel protein group. On the other hand, the two proteins were equally effective in producing improvement in the serum-protein concentration. The increase from the marked hypoproteinemia of 3.02% (based on whole blood) to 3.70 and 3.66% for the casein and fish protein groups is highly significant from a statistical standpoint and indicates that a marked progress toward a return to a normal level had already occurred in 7 days.

The total plasma volumes were quite similar in the three groups. When based on the quantity per 100 gm. of body

TABLE 3

*The comparative effect of casein (group II), mackerel protein (group III), and the basal low protein diet (group I) when fed over a 7-day period on the body weight, plasma volume, hemoglobin and plasma protein of male rats previously depleted by a prolonged low protein diet.*

GROUP	NO. OF TESTS	AVERAGE BODY WT. IN DEPLETION PER.			SUPPLEMENTED PERIOD				BLOOD VALUES AFTER SUPPLEMENT			
		Start	After 35 days	Loss	Average body weight		Change in body wt.	Total food eaten	Plasma vol. <sup>1</sup>		Hemoglobin <sup>2</sup>	Plasma proteins <sup>2</sup>
		gm.	gm.	%	After 3 days	After 7 days	gm.	gm.	Per 100 gm.	ml.	%	%
I	14	286.0	161.0	43.8	157.7	156.3	— 4.7	69.5	6.25(9)	10.25	8.37 ± 0.23 (6.80 - 9.82)	3.02 ± 0.08 (2.51 - 3.48)
II	16	285.0	163.3	42.7	168.2	178.8	+ 15.5	88.2	4.59(6)	9.04	9.83 ± 0.40 (7.32 - 12.57)	3.70 ± 0.07 (3.19 - 4.42)
III	15	294.0	170.0	42.2	184.2	194.1	+ 24.1	104.8	5.21(11)	10.42	10.42 ± 0.24 (8.39 - 11.80)	3.66 <sup>4</sup> ± 0.12 (2.84 - 4.45)
											6.28 <sup>2</sup>	4.44 <sup>2</sup>
											1.26 <sup>2</sup>	0.29 <sup>2</sup>

<sup>1</sup> The values in parentheses are the number of experiments on which satisfactory determinations of blood volume were completed.

<sup>2</sup> Including the Standard Error of Mean calculated as follows:  $\sqrt{2d^2/n} / \sqrt{n}$ .

<sup>3</sup> Mean Difference/Standard Error of Mean Differences when group is compared with group I. When this ratio exceeds 3.00, it is considered that the differences are statistically significant.

<sup>4</sup> This average based on only 13 determinations.

<sup>5</sup> Comparison of M.D./S.E.M.D. between groups II and III.

weight, the mean was highest in the basal control group (6.25), intermediate in the fish protein group (5.21) and lowest in the casein rats (4.59). The fact that the discrepancies become small when based on the total plasma volume without reference to body weight would indicate that the rapid variations in weight occurring during the supplement period were not accompanied by similar rapid adjustments in plasma volume.

Although no values of plasma volume are reported on control rats on a diet containing an adequate protein level throughout the depletion period, they are quite similar to those obtained with normal rats of our stock colony by Hechter ('45). Moreover, no alterations in value are evident when protein is administered for a week. On the other hand, Metcoff, Favour and Stare ('45) have indicated a significant decrease in plasma and blood volumes in acute protein deficiency.

#### DISCUSSION

All the experimental evidence is in agreement that the mixed mackerel muscle proteins have a considerably higher biological value than casein. Tuna proteins when fed at 9 and 12% and sardine proteins at 15% also gave considerably more rapid growth in weanling rats than diets containing similar amounts of casein. One should probably expect such a superiority for a mixed protein over a homogenous protein as casein as the deficiencies of one protein may to a considerable extent be supplemented by the other proteins.

The evaluation of biological activity by the Cannon method may well prove to be a more quantitative procedure than the usual growth method. It would appear to the authors that the factor for comparison should not be the weight increase over the 7-day period but rather this value plus the weight deficit of the control unsupplemented rats during the same interval.

The ratio of biological activity of fish protein to casein based on weight increases of the hypoproteinemic rats over a 7-day period would be on the first basis 1.55 (24.1 : 15.5) while by the second method it would be 1.43 (28.8 : 20.2).

On the basis of hemoglobin increase, it would also appear that the mackerel protein was somewhat superior to casein. The hemoglobin in the control group (8.37%) indicates that the prolonged protein-low diet had resulted in a severe anemia. This level was increased to 9.83 in the rats which received casein and to 10.42% for those animals which received the fish protein diet. Although the hemoglobin averaged considerably higher in the fish protein series than in the casein group, the differences are not significant from a statistical standpoint because of the relatively high variability in the individual experiments.

The plasma protein figure for the control rats is distinctly subnormal. Casein and mackerel protein were both equally effective in causing an increase in this value, the averages after the supplement period being 3.70 and 3.66%, respectively.

The fact that mackerel protein may be superior to casein in hemoglobin formation while it shows no superiority in causing the regeneration of the serum proteins may be explained in several ways. Either its amino acid makeup favors hemoglobin synthesis or as Whipple ('42) suggests, hemoglobin regeneration has a priority over the formation of plasma protein even though the amino acid distribution may be less suited to the former. Further comparative tests on rats in which each of these factors is varied separately should answer this question. It would also be of considerable interest to compare the biological efficiency of the single proteins making up the mixtures of the fish proteins used here.

#### SUMMARY

The mixed proteins of mackerel, sardine and tuna muscle were shown to afford superior growth to casein in weanling rats when fed at levels which give suboptimal growth with casein.

The superiority of mackerel protein is further attested by the fact that it causes greater recovery in weight and a more pronounced stimulation in hemoglobin regeneration than casein in rats rendered hypoproteinemic and tested by the

method of Cannon. Mackerel protein proved to have a potency equal to casein in causing a regeneration of plasma protein.

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# STUDIES ON THE NUTRITIVE VALUE OF FISH PROTEINS

## II. THE USE OF MACKEREL PROTEIN IN THE BIOASSAY TEST FOR VITAMIN A

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Casein has usually been the protein of choice which is incorporated in the diets used in the bioassay tests to determine vitamin A. In fact, it is prescribed in the U.S.P. diet to be used for vitamin A assay. This is presumably because of its ready availability and the ease with which it may be rendered vitamin A-free. Moreover, it is a protein which possesses a high nutritive value when fed at a level of 18% in the diet.

Although it is well known that growth cannot be brought about when any single essential component of the diet is absent, the present experiments afford an opportunity to determine the effect of a protein other than casein when fed as a constituent of a diet limited in vitamin A. The protein used in these experiments was mackerel protein which possesses a biological activity considerably in excess of casein (Deuel et al., '45). Moreover, by its method of preparation it would be expected to be free of vitamin A and this was also indicated by spectrophotometric analysis. Likewise, it is a protein potentially available in unlimited amounts.

### METHODS AND RESULTS

The bioassay tests were carried out by the U.S.P. XII method except that the basal diet in one group was modified by

the substitution of mackerel protein for casein. In series II where the protein was in some cases reduced to 9%, it was replaced by the addition of a corresponding amount of starch. In series III rats were started on the bioassays as soon as they were sufficiently depleted when the average length of depletion period was about 17 days instead of 20 days prescribed as the minimum in the U.S.P. method.

Three series of tests were made on 222 rats. Series I and II were conducted on rats from the U.S.C. colony and series III with animals of the Sprague-Dawley strain. In series II, the animals were started on the respective diets at weaning while in the other tests, the experimental diets were employed only during the assay period. The vitamin A supplements were made from U.S.P. Reference cod-liver oil. They were made up in cottonseed oil with 5 mg. of alpha tocopherol added per milliliter. A total of 0.1 ml. of the supplements was administered daily. The negative controls in each series were given 0.1 ml. of the cottonseed oil-tocopherol mixture daily. Litter mates were distributed between the various groups. Animals were considered sufficiently depleted to start on the assay period when their weight was stationary for 5 days or after a shorter period if a marked drop in weight (more than 6 gm.) occurred. Food was given *ad libitum*. The amount of the food consumption was determined in the second series. The results of the tests are summarized in table 1.

#### DISCUSSION

A commercial mackerel protein, prepared by isoelectric precipitation followed by purification by solvent extraction, would appear to be perfectly adequate for use in the bioassay test for vitamin A in place of casein. In the first place, it is extremely low in vitamin A so that depletion occurs when it is fed from weaning in a similar period to casein (16.7 days for the fish protein compared with 17.6 days for the control casein rats in our tests). Also, in all cases as large a proportion of the negative controls died during the tests with the fish protein

TABLE 1  
The effect of different dietary proteins on the growth of rats receiving given amounts of vitamin A.

SERIES NO.	BASAL DIET		VITAMIN A ADMINISTERED DAILY		NUMBER OF EXPERIMENTS		ASSAY PERIOD						
	Protein	Per cent protein	Per cent cysteine	I.U.	Male rats	Fe-male rats	Body weight <sup>1</sup>		Av. weight increase		Av. daily food intake gm	Average day of death <sup>2</sup>	
							Start	End	Male	Fe-male			Total <sup>1</sup>
USC rats													
I	Casein	18	..	1.5	5	7	85.0	103.7	10.2	24.4	17.3 (11)	15 (1)	
	Mackerel	18	..	1.5	5	7	87.8	106.6	18.4	19.5 (9)	19.0	14.7 (3)	
	Casein	18	..	3.5	3	6	91.8	141.6	60.5	39.3	49.9	..	
	Mackerel	18	..	3.5	4	5	86.6	139.2	62.0	43.3	52.6	..	
	Casein	18	..	0.0	7	6	89.5	..	..	..	24.0 (2)	19.3 (11)	
II	Mackerel	18	..	0.0	6	7	81.7	..	..	..	14.0 (3)	19.6 (10)	
	Casein	18	..	2.0	6	8	99.7	148.6	56.6	41.2	48.9	..	
	Mackerel	18	..	2.0	3	7	92.1	145.3	62.0	44.3	53.2	..	
	Casein	9	..	2.0	3	5	100.5	147.1	52.0	41.3	46.6	..	
	Mackerel	9	..	2.0	7	6	88.4	141.6	62.6	43.7	53.2	..	
III	Casein	9	1	2.0	7	6	102.6	153.3	56.0	45.3	50.7	..	
	Casein	9	1	0.0	7	6	98.8	..	..	..	..	..	
	Mackerel	18	0	0.0	8	6	101.4	..	..	..	..	..	
	Casein	18	..	1.2	5	6	69.6	107.8	43.0	33.3	38.2	21.2 (8)	
	Mackerel	18	..	1.2	5	7	67.4	123.9	59.0	50.0	54.5	18.0 (8)	
Sprague-Dawley rats													
III	Casein	18	..	3.5	4	7	71.2	136.0	68.5	50.4	59.4	..	
	Mackerel	18	..	3.5	5	7	71.8	149.8	86.4	69.5	78.0	..	
	Casein	18	..	0.0	5	6	74.5	..	..	..	..	..	
	Mackerel	18	..	0.0	4	8	69.2	..	..	..	..	..	
	Casein	18	..	..	..	..	..	..	..	..	..	..	

The figures in parentheses indicate the number of experiments in the average.

as with the casein and the average day of death for the decedents was slightly less than for casein.

The extent of growth during the 28-day bioassay period when vitamin A was given was significantly greater with the mackerel protein in the experiments where Sprague-Dawley rats were employed. The growth of such rats receiving the fish protein exceeded that of the rats on the standard casein diet by 43 and 31% where vitamin A was administered at levels of 1.2 and 3.5 I.U., respectively. On the other hand, with the U.S.C. strain the differences in growth on the two proteins were not significant although in each case the increase in weight with the group receiving the mackerel protein somewhat exceeds that of those receiving casein. When 1% of cystine was added to the diet containing 9% casein (Mendel, '15), the growth was slightly though not significantly increased over that obtained where this amino acid was omitted. This addition had no effect on the survival time or on the proportion of rats which died in the negative control group where no vitamin A was administered. The increased growth response of the mackerel protein over casein obtained with series III is supported by the results of the earlier experiments where a more pronounced growth was shown to obtain with mackerel protein even when the proteins were fed in a complete diet at a level of 21% to weanling rats (Deuel et al., '45).

These experiments are of interest in relation to the recent report of Dye, Bateman and Porter ('45) who were unable to demonstrate any increased growth response to vitamin A when casein was fed at levels of 9, 18 and 36%. However, these experiments are not directly comparable to ours inasmuch as paired feeding was employed while in the present tests the animals were fed ad libitum. It is also true that although greater growth might occur on the higher protein level when the net caloric intake is identical with that on the lower level of protein, such results can only be attained when the food consumption is increased sufficiently with the high protein diets to compensate for the larger loss due to the specific dynamic action of the protein. It would appear if an

identical growth occurs on isocaloric diets with low and high protein that a greater efficiency characterizes the latter case. A smaller net balance of calories is available in the latter case after that required for basal metabolism and specific dynamic action has been deducted from the total.

It is evident that not only the caloric intake (Muelder and Kelly, '41) but also the quality of the protein may influence the growth response to given amounts of vitamin A. If limited quantities of food are taken as may be the case on diets where minimum amounts of vitamin A are available, there is less chance that a protein deficiency might occur when a protein of higher biological value than casein is employed. In addition, other growth factors are involved inasmuch as it has been shown that the amount of growth hormone available will influence such a growth response (Ershoff and Deuel, '45) when administered with limited amounts of vitamin A.

#### SUMMARY

A commercially prepared mackerel protein powder has been shown to be a satisfactory protein to use in bioassay experiments for the determination of vitamin A.

This protein contained no detectable quantity of vitamin A as indicated by the fact that the average number of rats in the control groups surviving the 28-day test period as well as the average survival period of the decedents compare favorably with the casein tests.

When uniform doses of vitamin A were administered daily, greater growth was obtained in rats receiving the fish protein diet than in litter mates which were fed the corresponding casein diet. The differences were statistically significant in the Sprague-Dawley rats but not in those of the U.S.C. strain. This would indicate that greater growth may result with limited amounts of vitamin A when a protein of higher nutritive value than casein is employed.

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## THE AVAILABILITY OF WHEAT BRAN PHOSPHORUS FOR THE RAT <sup>1</sup>

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Patten and Hart ('04) showed that practically all of the phosphorus of wheat bran is in the form of salts of phytic acid. Hart, McCollum and Fuller ('09) demonstrated that wheat bran could serve as the main source of available phosphorus for the skeletal development of young swine. Pigs on a low phosphorus basal ration supplemented with wheat bran produced thigh bones with an ash content of 53 to 54%, while those receiving precipitated calcium phosphate as the phosphorus supplement, and at the same phosphorus level, produced thigh bones with an ash content of 46 to 55%. On the basal ration the ash content was 31 to 33%. Approximately 81% of the total phosphorus of the bran ration was phytic acid phosphorus. The pigs had access to sunlight.

Hart and coworkers ('27) observed that cattle in a phosphorus deficient area of Wisconsin were completely protected from rickets by the addition to the ration of either wheat bran or bone meal. Both products served to contribute phosphorus to the ration. These animals also had access to direct sunlight during the experimental period.

All of the above studies pointed toward the availability of the phytic acid phosphorus of wheat bran for swine and cattle,

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at least where this product was fed under practical conditions with sunlight available. Since these observations were made, there has accumulated definite evidence that in the absence of vitamin D, the phosphorus of isolated phytic acid is poorly available to the rat (Krieger, Bunkfeldt, and Steenbock, '40). However, it was also shown by Krieger and coworkers ('40, '41) that in the presence of vitamin D there is a marked improvement in the utilization of isolated phytic acid phosphorus. In studies with chicks, Lowe, Steenbock and Krieger ('39) and McGinnis, Norris, and Heuser ('44) showed that the phosphorus of cereals and legumes was poorly available in the absence of vitamin D, and the latter group found phytin-phosphorus nearly as available as inorganic phosphorus in the presence of 160 A.O.A.C. units of vitamin D per 100 gm. of diet.

McCance and Widdowson ('35) have pointed out that because approximately 40 to 70% of the phosphorus of cereal grains is in the form of phytic acid, cereals and cereal products are a poor source of available phosphorus for man. From their experiments with human subjects they concluded that when cereals constitute the largest part of the diet, the low availability of phytin phosphorus can become a serious matter.

It has been suggested by Hart et al. ('09), Lowe and Steenbock ('36), and Singsen and Mitchell ('44) that ingestion of the enzyme phytase with the food may be necessary for the efficient utilization of phytin phosphorus. Thus the observation of Krieger, Bunkfeldt and Steenbock ('40) that the phosphorus of isolated phytic acid fed in a purified diet is of poor availability might be explained by a lack of dietary source of phytase. Likewise, the low availability of the phosphorus of cereals observed by Bruce and Callow ('34) for rats and by McCance and Widdowson ('35) in man might be similarly related to an inadequate dietary source of phytase since cooked cereals were used in part. Singsen and Mitchell ('44) found that the efficiency of the chick in utilizing the phytin phosphorus of soybean meal was satisfactory when field cured alf-

alfa meal was included in the ration as a source of phytase. Dehydrated alfalfa meal was not effective, presumably because the phytase had been destroyed during processing. However, the validity of this interpretation is open to question since the field cured alfalfa hay supplied extra vitamin D as well as phytase. A feeding oil furnished vitamin D at a level calculated to be 100 A.O.A.C. units per 100 gm. of ration, which may have been inadequate. Spitzer and Phillips ('45a, b) concluded that a dietary source of phytase was not essential for the utilization of the phytin phosphorus of soybean meal by the rat.

With these facts in mind, two experiments were made with rats to study the effect of dietary phytase and vitamin D on the availability of the phosphorus of wheat bran.

#### EXPERIMENTAL

The basal ration was similar to the low phosphorus diet developed by Schneider and Steenbock ('39) and had the following composition: cerelose (glucose monohydrate) 50, dextrin 20, fibrin<sup>2</sup> 20, phosphorus-free salts 4, cottonseed oil (plus 100 µg.  $\beta$ -carotene per gram) 5, and 1 to 20 liver concentrate 1. Water soluble vitamins were incorporated in the ration so that every 100 gm. of ration contained 0.5 mg. thiamine, 0.5 mg. riboflavin, 0.6 mg. niacin, 0.6 mg. pyridoxine, 5.0 mg. calcium pantothenate, 30.0 mg. p-aminobenzoic acid, 100.0 mg. inositol, and 250.0 mg. choline.

The phosphorus-free salts were prepared by substituting calcium lactate and an equimolecular mixture of KCl and  $\text{KHCO}_3$  for the  $\text{CaHPO}_4$  and  $\text{K}_2\text{HPO}_4$  of salts IV (Hegsted, Mills, Elvehjem and Hart, '41). The substitutions were equimolecular with respect to Ca and K.

The raw wheat bran as well as the commercial bran cereal<sup>3</sup> contained from 1.39 to 1.40 gm. of phosphorus<sup>4</sup> per 100 gm.

<sup>2</sup> The use of fibrin in low P rations was suggested by Jones ('38).

<sup>3</sup> Kellogg's All-Bran.

<sup>4</sup> Phosphorus determinations were made by the method of Fiske and Subbarow ('25). Measurements were made in the Evelyn colorimeter.

and was incorporated in the phosphorus-free basal ration in place of cerelose at a level of 20% to furnish 0.28 gm. of phosphorus per 100 gm. of ration. This made the test diets identical in phosphorus content with a control ration in which 4% of salts IV were substituted for the phosphorus-free salts. A low phosphorus control ration was made by replacing the test bran with a washed, phosphorus-free bran. In this way, an approximate equivalence in fiber was maintained. The washed bran was prepared by incubating raw wheat bran with water at 37°C. for 24 hours to allow the phytase to liberate inorganic phosphorus. After filtering, washing and drying, the washed bran contained 0.02% of phosphorus. Consequently it contributed 0.004 gm. of phosphorus per 100 gm. of ration at the 20% level. The fibrin contributed approximately 0.02 gm. of phosphorus per 100 gm. of ration.

All of the rations contained 0.60% of calcium. The 4% of salts IV as well as the phosphorus-free salts contributed 0.59 gm. of calcium to each 100 gm. of ration and the bran added 0.01 gm. A calcium to phosphorus ratio of 2 existed in all experimental diets.

Male albino rats of the Sprague-Dawley strain 32 days of age were divided into groups of 4 with an average weight of 68 gm. Each rat was housed in an individual raised bottom cage. The daily allotment of food was equalized for all rats except those fed the phosphorus-free ration. After 4 weeks the femurs of each animal were removed and the ash of individual bones was determined by the official A.O.A.C. procedure for the rat.

In the first experiment the effect of vitamin D on the utilization of the phosphorus of the phytic acid of wheat bran was determined. A prepared bran cereal, Kellogg's All-bran, was chosen for the test because it represents a commercial product high in phytic acid with no phytase activity.

The following rations were fed to groups of four rats:

Group 1. Low phosphorus control, no vitamin D, washed bran, phosphorus-free salts.

Group 2. Inorganic phosphorus control, 250 I.U. of vitamin D<sup>6</sup> per rat each week, salts IV, washed bran.

Group 3. Test bran without vitamin D, phosphorus-free salts.

Group 4. Test bran plus 70 I.U. of vitamin D<sup>6</sup> per 100 gm. of ration, phosphorus-free salts.

Group 5. Test bran plus 140 I.U. of vitamin D<sup>6</sup> per 100 gm. of ration, phosphorus-free salts.

Group 6. Test bran plus 210 I.U. of vitamin D<sup>6</sup> per 100 gm. of ration, phosphorus-free salts.

Group 7. Test bran plus 280 I.U. of vitamin D<sup>6</sup> per 100 gm. of ration, phosphorus-free salts.

The second experiment was designed to study the effect of a dietary source of the phosphate liberating enzyme, phytase, on the utilization of phytic acid in wheat bran. The sample of raw wheat bran (also used in the first experiment for the preparation of the washed bran) was found to contain an acid phosphatase. A portion of the bran was autoclaved at 15 pounds pressure for 1 hour to destroy enzyme activity. Rations were made from these two samples of bran similar in composition and phosphorus content to those fed in the first experiment. The plan of the test was as follows (a third control ration, group 2, was included in this experiment):

Group 1. Low phosphorus control, no vitamin D, phosphorus-free salts, washed bran.

Group 2. Inorganic phosphorus control, no vitamin D, salts IV, washed bran.

Group 3. Inorganic phosphorus control, 250 I.U. vitamin D per rat each week,<sup>5</sup> salts IV, washed bran (repetition of group 2 in experiment 1).

Group 4. Raw wheat bran, no vitamin D, phosphorus-free salts.

Group 5. Raw wheat bran with 250 I.U. of vitamin D per rat each week,<sup>5</sup> phosphorus-free salts.

<sup>6</sup> Drisdol. Winthrop Chemical Co. Irradiated ergosterol in propylene glycol. This supplement was given by pipette.

<sup>5</sup> Irradiated ergosterol was incorporated in the ration.

Group 6. Autoclaved wheat bran, no vitamin D, phosphorus-free salts.

Group 7. Autoclaved wheat bran plus 250 I.U. of vitamin D per rat each week,<sup>5</sup> phosphorus-free salts.

The determination of phytase activity was made according to the method of Spitzer and Phillips ('45b). A 50-mg. sample of raw wheat bran liberated 110  $\mu$ g. of inorganic phosphorus when incubated for 2 hours with a sodium phytase substrate containing 230  $\mu$ g. of phosphorus. This value was corrected for the inorganic phosphorus present at zero time and for the phosphorus liberated during the 2 hours from substrates carried in the enzyme preparation. A 200-mg. sample of either the autoclaved bran or the commercial bran cereal showed no phytase activity.

### RESULTS

The growth data together with the per cent of ash of the femurs are given in tables 1 and 2. The rats on the low phosphorus control diet grew slowly and reached a maximum

TABLE 1

*Record of growth and bone ash in the first experiment.*

SOURCE OF PHOSPHORUS	NONE	IN- ORGANIC	COMMERCIAL BRAN CEREAL				
VITAMIN D/100 GM. RATION	None	Excess	None	70 I.U.	140 I.U.	210 I.U.	280 I.U.
GROUP NUMBER	1	2	3	4	5	6	7
Average initial weight	69	68	70	66	67	67	67
Average final (4 wks.) weight	110	156	154	153	162	150	149
Average bone ash	24.7	57.4	36.4	46.3	50.6	49.2	48.3
Mean deviation	1.1	0.57	1.7	1.5	1.3	1.1	1.8

weight between the third and fourth week when weight losses became evident. Food consumption dropped to about 6 gm. per rat per day during the fourth week. Those rats fed rations containing phosphorus grew 20 to 25 gm. a week and consumed up to 16 gm. of ration a day by the end of the ex-

periment. The food allotment was limited in both studies to the amount eaten by the rats fed the raw bran ration without vitamin D.

In the first experiment a bone ash of 24.7% was found for animals fed the phosphorus-free ration. The addition of 0.28% of inorganic phosphorus and adequate vitamin D to this ration allowed development of bones of 57.4% ash. These findings were repeated in the second experiment, and in addition it was found that even in the absence of vitamin D, rats fed this ration developed femurs of 54.4% ash content. However, when the 0.28% of phosphorus was contributed to the

TABLE 2

*Record of growth and bone ash in the second experiment.*

SOURCE OF PHOSPHORUS	NONE			INORGANIC		RAW BRAN		COOKED BRAN	
VITAMIN D	None	None	Excess	None	Excess	None	Excess	None	Excess
GROUP NUMBER	1	2	3	4	5	6	7		
Average initial weight	68	69	68	68	69	68	68		
Average final (4 wks.) weight	110	169	176	163	165	166	165		
Average bone ash	21.4	54.4	56.7	32.0	48.8	38.9	50.4		
Mean deviation	0.90	1.5	1.0	1.2	1.7	3.3	1.0		

ration by the commercial bran without added vitamin D, a bone ash of 36.4% resulted. This value was increased to about 50% by the addition of vitamin D to the ration.

In the presence of vitamin D, both the autoclaved and the raw wheat bran gave a bone ash picture similar to that obtained with the commercial bran. In the absence of vitamin D, the bone ash of rats fed raw bran was 32.0%; values of 38.9 and 36.4% resulted from the same bran cooked and the commercial bran cereal.

#### DISCUSSION

The presence or absence of the enzyme phytase in the diet had no apparent effect on the availability of phytin phosphorus. In fact, the bone ash values of the rats fed the raw

bran supplement, which contained phytase, were not as great as those of the rats receiving inactivated bran. This indicates a partial hydrolysis of phytin during cooking. It is probable that the phosphorus splitting enzymes elaborated by the intestinal mucosa (Spitzer and Phillips, '45b) and by the flora of the gastro-intestinal tract are the critical means by which phytin is hydrolyzed in the intestine of the rat.

The absolute amounts of calcium and phosphorus in these rations were adequate (Nicolaysen, '37) and a calcium to phosphorus ratio of 2 was maintained. Since about 85% of the phosphorus in these diets was in the form of phytin (Patten and Hart, '04), the importance of vitamin D is clearly demonstrated. The bone ash values of the rats receiving phytin phosphorus and adequate vitamin D were only 4 to 7% lower than those of the rats receiving the inorganic phosphorus supplement. The low availability of the phytin phosphorus of cereals has been overemphasized in the literature. As a result it is often not realized that satisfactory bone development is possible with diets carrying only a normal amount of phosphorus mainly in the form of phytin.

These data support the results of Krieger et al. ('40) who found that vitamin D aided in the utilization of the phosphorus of isolated phytic acid by the rat, and of McGinnis et al. ('44) who showed the importance of vitamin D for the utilization of phosphorus in cereals and legumes by the chick. Further, it appears from our data that there may be a direct relationship between the level of phytic acid phosphorus in the ration and the vitamin D requirement.

#### SUMMARY

1. A study of the availability of phytin phosphorus was made with the rat using diets of normal calcium and phosphorus levels. About 85% of the dietary phosphorus was in the form of phytic acid and its salts, as carried by wheat bran.

2. In the absence of vitamin D, the phytin phosphorus of wheat bran was poorly utilized. An adequate intake of this

vitamin increased the utilization nearly to that of inorganic phosphorus as measured by bone ash.

3. The presence or absence of phosphorus splitting enzymes in the diets did not alter the apparent availability of phytin phosphorus.

#### NOTE

After this paper was prepared for The Journal of Nutrition an article by Singsen and Mitchell appeared (Poultry Sci., vol. 24, p. 479, 1945) in which is found confirmation of the idea that the availability of phytin phosphorus is related to the amount of vitamin D in the ration.

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# RELATION OF FAT TO ECONOMY OF FOOD UTILIZATION

I. BY THE GROWING ALBINO RAT <sup>1</sup>

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Fat, as a dietary component, is characterized by several unique capacities. In addition to being the nutrient of maximum energy value it confers palatability and "staying quality" to diets. It serves important purposes as a carrier of vitamins A, D and E, and provides the apparently essential linoleic or linolenic acids. It contributes to the composition of tissue lipids; it insulates, supports and cushions vital organs; as a nutritive reserve it insures the availability of energy as needed; and it diminishes the energy expense of utilization of the nutrients with which it is associated.

In spite of the existing knowledge of these functions of the fats, however, the student of nutrition has still to deal with a popular understanding that, for practical purposes, fat and carbohydrate may be regarded as interchangeable in accord with their metabolizable energy values, fat therefore being approximately 2.25 times as potent as carbohydrate for the same purposes.

During recent years a volume of evidence has accumulated as to the utilization of fats and carbohydrates, individually and in combination with other nutrients, and especially as affected by associated vitamins, which emphasizes the complication of conditions which determine the relative values of

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these nutrients, and warrants the conception of the facts in this matter as determined, in large part at least, by the relative extent to which the requirements of fat and of carbohydrate utilization are satisfied by any nutritive ensemble in question.

It thus becomes clear that the only logical background for the determination of the interrelations of fats and carbohydrates in nutrition is one which affords optimum conditions for the utilization of each.

However, it is still necessary, for purposes of guidance in nutritional affairs, to observe the facts in this matter as they are encountered under conditions of dietary practice.

Forbes and Swift ('44) recently demonstrated that fat decreases the energy expense of utilization of the food protein and carbohydrate with which it is combined. This paper and the following now report the results of two further studies of the functions of fat, one with growing and the other with mature albino rats as subjects, both groups having received the same diets containing 2, 5, 10 and 30% of fat, respectively; these diets were rendered isocaloric by compensating adjustment of carbohydrate contents, and so compounded and fed that each supplied gross energy, protein, and vitamins in the same proportion.

The first of these experiments, with weanling rats as subjects, was conducted by the so-called body balance method of this laboratory in accord with which the rats are allowed normal freedom of movement, and in which a single measurement of the heat production for a period of 70 days is made by subtraction of the energy of the excreta and of the body increase from the gross energy of the food.

In connection with this procedure values were determined for the digestibility of the diets, and for the retention of energy and of nitrogen, the observed differences in nutrient utilization being considered as resultants of the differences in the metabolism of energy and protein, in the dynamic effects of the diets, and in the physical activity of the subjects.

The second of these experiments, with mature rats as subjects, was conducted by the open-circuit Haldane procedure, with voluntary activity excluded. The heat increment or dynamic effect of the food was determined as the difference in the amounts of heat produced from a basal and a supplemented diet, both of the same composition, and containing 2, 5, 10 or 30% of fat.

The first of these experiments, with weanling rats as subjects, is discussed in the present, and the second, with mature rats as subjects, in the following paper.

#### EXPERIMENTAL

The diets <sup>2</sup> were compounded as indicated in table 1. The amounts of vitamins incorporated in the diets were determined in consideration of evidence presented by Burr and

TABLE 1  
*General composition of diets.*

	DIET NO. 1	DIET NO. 2	DIET NO. 3	DIET NO. 4
	%	%	%	%
Salt mixture <sup>1</sup>	4.00	4.00	4.00	4.00
Corn oil <sup>2</sup>	2.00	2.00	2.00	2.00
Protein mixture <sup>3</sup>	31.40	32.82	35.19	44.67
Carbohydrate mixture <sup>4</sup>	62.60	58.18	50.81	21.33
Lard	0.00	3.00	8.00	28.00
Calories per gm.	4.058	4.241	4.548	5.773
Isocaloric factors	1.0000	.9567	.8922	.7029

Vitamin supplements were added in the following amounts per kg. of Diet 1, and to equivalent quantities of the other diets: carotene 6.0 mg., thiamine-HCl 5.0 mg., riboflavin 5.0 mg., pyridoxine-HCl 6.25 mg., niacin 6.25 mg., calcium pantothenate 50 mg., choline chloride 400 mg., para-aminobenzoic acid 95 mg., 2-methyl-1,4 naphthoquinone 2.5 mg., alpha-tocopherol 100 mg., and inositol 2.0 gm. Vitamin D was supplied in irradiated yeast in the amount of 47.1 gm. per kg. of the 2% fat diet and of isocaloric quantities of the other diets.

<sup>1</sup> U.S.P. XII no. 2.

<sup>2</sup> Considered to furnish sufficient essential fatty acid.

<sup>3</sup> Casein 50%, skim milk powder 25%, irradiated yeast 15%, brewer's yeast 10%. Mixture contained 10.19% N.

<sup>4</sup> Corn starch 34%, sucrose 33%, dextrin 22%, and dextrose (cerelease) 11%.

<sup>5</sup> The authors gratefully acknowledge the supply of the vitamin components of the diets by Merck and Company.

Burr ('29), Evans and Emerson ('43), Griffith and Farris ('42), Martin ('39), and Rosenberg ('42).

Diet no. 1 was made to contain 20% of protein. The other three diets were compounded by means of an algebraic computation based on the nitrogen and energy contents of the non-nitrogenous nutrients to such effect that the ratio of the energy of the protein to the energy of the diet as a whole was constant in all four diets. It was then possible, by the use of the so-called isocaloric factors in table 1, to feed the four diets so as to supply the same amounts of protein and energy to each rat.

TABLE 2

*Constituents of diets of different fat and different carbohydrate contents which supply the same quantities of protein and of energy.*

DIET NO.	APPROXIMATELY ISOCALORIC QUANTITIES OF DIETS	PROTEIN		FAT <sup>1</sup>		CARBOHYDRATE <sup>2</sup>		ENERGY	
		%	gm.	%	gm.	%	gm.	cal./gm.	Cal.
1	10.00	20.00	2.000	2.07	.207	67.78	6.788	4058	40.58
2	9.57	20.90	2.000	5.07	.485	63.68	6.654	4241	40.57
3	8.92	22.41	2.000	10.08	.899	57.66	6.464	4548	40.58
4	7.03	28.45	2.000	30.10	2.115	32.70	4.651	5773	40.58

<sup>1</sup> Fat added as corn oil and lard plus a very small amount in the protein mixture.

<sup>2</sup> Carbohydrate added as carbohydrate mixture and 22.75% of the protein mixture.

Table 2 gives the quantities of the diets required to supply protein and energy each at a constant rate; and also the protein, fat, carbohydrate and energy contents of isocaloric portions of the diets.

The proportions of fat to carbohydrate in diets nos. 1, 2, 3 and 4 were 1:32.8, 1:13.7, 1:7.19 and 1:2.20, respectively; and the reciprocal proportions of carbohydrate to fat in diets nos. 1, 2, 3 and 4, were 1:.031, 1:.073, 1:.139, and 1:.455, respectively.

While these ratios constitute the really significant bases for conclusions from the investigation, the diets will be designated, for convenience, by their approximate fat contents—to wit, 2, 5, 10 and 30%.

The experimental subjects were ten litter-fours of weanling albino rats subdivided in a manner providing four groups of ten individuals, each such group containing one rat from each of ten litters.

The rats were fed once a day, the amount given to each rat of a litter-four being determined by the individual of the four which ate the smallest amount of food. By this method of food control the caloric and the nitrogen intakes of each group of ten rats were maintained identical. The palatability of the diets increased in the order of their fat content, there being a total of thirty-two feed refusals by the rats receiving the 2% fat diet and only three by the rats receiving the 30% fat diet.

The urines were collected daily, and two aliquots were prepared, one preserved with 25 ml. of 1.7 Sp. Gr.  $\text{H}_2\text{SO}_4$  per 5-pint bottle for the determination of nitrogen, while the other was dried for the determination of energy by means of the bomb calorimeter. The feces were also collected daily, and after drying at  $50^\circ\text{C}$ ., were allowed to come into moisture equilibrium with the air before being weighed and ground for analysis.

This experiment was conducted at an environmental temperature thermostatically controlled at  $29.5^\circ\text{C}$ .

The bodies of the rats were cut up with shears, and were prepared for extraction by drying over sulphuric acid in vacuum desiccators at room temperature. After extraction with ether the residues were ground in an Excelsior mill, which reduced them to a condition sufficiently finely divided for sampling and determination of nitrogen and energy.

Table 3 gives the average weekly live weights of the four groups of ten rats, each of which groups received one of the experimental diets during the 10 weeks of the experiment.

An inspection of these average weekly weights for ten rats shows that the capacities of the diets to produce gain in weight were in the increasing order of their fat contents. During the third to the ninth week the rats which received diet no. 4, containing 30% of fat, were 3, 4, 7, 11, 14 and 16

gm. heavier than the rats which received the same amounts of energy in diet no. 1, containing 2% of fat. Since each rat of a litter-four was held to the same caloric intake, the rats were in effect penalized in accord with the amount that they grew, by virtue of the fact that the greater the growth the greater is the maintenance requirement. The observed differences in gain in weight produced by the four diets, therefore, were smaller than they should have been if the basis of comparison had been perfectly equitable.

TABLE 3

*Average liveweights of rats during 10 weeks on isocaloric quantities of diets containing 2, 5, 10 and 30%, respectively, of fat.*

DIET NO.	FAT CONTENT OF DIET	INITIAL BODY WEIGHT	WEEK NUMBER									
			1	2	3	4	5	6	7	8	9	10
			gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1	2	48.5	63	93	114	134	154	169	184	200	210	220
2	5	48.2	63	93	115	136	157	174	191	207	220	231
3	10	47.2	62	94	117	136	158	176	194	210	225	235
4	30	47.7	62	93	117	138	161	180	198	216	230	239

The average amounts of food and food nitrogen eaten per rat during 70 days by each of the groups of ten, and the average initial and final weights of body nitrogen and of body fat, are set forth in table 4. The gains in body nitrogen and body fat increased in the order of the increasing fat content of the diets, except that the increase in the body fat of the rats which received the 30% fat diet was not greater than that of the rats which received the 10% fat diet.

In regard to the cause of the increased fat contents of the rat bodies with increase in the fat content of the diets—this could logically be due either to a fundamental advantage in favor of the energy expense of utilization of fat, or to a difference in the effects of the dietary conditions, especially as to vitamin contents, as affecting the utilization of fat and carbohydrate. The intake of vitamins, however, is believed to have been adequate.

It is a fact, however, that the body residues from the ether extraction in the coarsely cut-up condition were not re-extracted after grinding. The weights of body fat, therefore, were not of the most critical significance.

The average gain of fat in the rats which received the 2% fat diet was 21.04 gm., while their food contained only 13.22 gm. Fat synthesis, therefore, took place from the diet containing the minimum amount of fat.

TABLE 4

*Average amounts of food eaten during 70 days; and initial and final nitrogen and fat contents of rat bodies.<sup>1</sup>*

FAT CONTENT OF DIETS	FOOD EATEN	INTAKE OF NITROGEN	BODY NITROGEN		BODY FAT	
			Initial	Final	Initial	Final
%	gm.	gm	gm.	gm.	gm.	gm.
2	703.4	22.507	1.17	6.86	3.72	24.75
5	672.9	22.507	1.16	7.24	3.69	26.96
10	627.6	22.507	1.14	7.50	3.62	27.79
30	494.4	22.507	1.15	7.59	3.66	27.73

<sup>1</sup> The composition of the bodies of the rats at the beginning of the experiment was computed from the directly determined composition of a control group; the composition of the rat bodies at the end of the experiment was determined by chemical analysis.

The per cent of recovery of feed nitrogen in the nitrogen of the urine, feces and body gain for the rats which received the 2, 5, 10 and 30% fat diets were 98, 98, 99 and 99, respectively.

The data in table 5 show that there were slight decreases in the nitrogen of the urine, much larger decreases in the nitrogen of the feces, and increases in the nitrogen retained, during the 70-day experiment, in the increasing order of the fat contents of the diets. The odds that the nitrogen retention from the 30% fat diet was higher than from the 2% fat diet were 253:1. Since the protein intake in all diets was the same, these observations signify that the increasing fat content of the isocaloric diets favored both the digestion and



the fundamental utilization of the protein present, the nitrogen retention from the 30% fat diet being 9.8% greater in amount than that from the 2% fat diet.

From the data in table 6 it is evident that the difference in the fat content of the diets was associated with only slight differences in the outgo of energy in feces, urine and heat, the odds that the heat production from the 30% fat diet was

TABLE 5  
*Partition of average nitrogen intake per rat during 70 days.*

FAT CONTENT OF DIETS	N I T R O G E N			
	Intake	Output in		Retained
		Urine	Feces	
%	gm.	gm.	gm.	gm.
2	22.51	14.34	2.06	6.11
5	22.51	13.95	2.03	6.53
10	22.51	14.10	1.86	6.55
30	22.51	14.10	1.70	6.71

TABLE 6  
*Partition of average daily intake of food energy per rat during 70 days.*

FAT CON- TENT OF DIET	GROSS ENERGY INTAKE	E N E R G Y I N T A K E				E N E R G Y O U T P U T			E N E R G Y RETAINED
		Protein	Carbo- hy- drate	Fat	Metabo- lizable	In feces	In urine	As heat	
%	Cal.	Cal.	Cal.	Cal.	Cal.	Cal.	Cal.	Cal.	Cal.
2	2854	799	1912	143	2601	128	125	2195	406
5	2854	799	1713	342	2605	126	123	2165	440
10	2854	799	1456	599	2610	121	123	2154	456
30	2854	799	657	1398	2615	123	116	2155	460

less than from the 2% fat diet being 59 to 1; but the differences in energy retained were much more significant, the odds being 253 to 1 that the amount of energy retained from the 30% fat diet was greater than that retained from the 2% fat diet. It is a coincidence, and not an error, that the odds, as stated, relating to nitrogen and energy retention were in both cases 253:1. Of the difference in the amounts of energy stored from the 2% and the 30% fat diets 54.6% was from protein.

Since the subjects of this experiment were allowed normal freedom of exercise, it was not determined whether differences in the amount of voluntary exercise taken by these growing rats on the isocaloric diets of different fat content contributed to the observed differences in heat production and in energy retention. Smith and Conger ('44), however, found that 56% of food calories coming from fat caused no disturbance in voluntary activity, and that as much as 72% in fat calories caused only slight depression of activity. In the 30% fat diet which was fed in the present investigation the fat supplied about 50% of the energy. Further evidence on this aspect of the problem was obtained from the experiment with mature rats as subjects, with voluntary activity excluded.

With respect to the utilization of both nitrogen and energy the increased efficiency of the 5% fat diet as compared with the 2% fat diet was much greater than that of the 30% fat diet as compared with the 5% fat diet.

#### SUMMARY

A 70-day metabolism and body analysis experiment was conducted to determine the effects of differences in the fat content of isocaloric diets on the utilization of food energy and protein.

The subjects were four groups of 10, growing male albino rats, each of these four groups containing one rat from each of the same ten litters.

A comparison was made of four diets containing 2, 5, 10 and 30% of fat, respectively, these diets being so compounded and fed as to supply to each rat of a litter-four the same quantities of gross energy, protein, and vitamins.

Determinations were made of gains in live weight, nitrogen, fat and energy, with a single value of the heat production for the 70 days as the energy of the food minus the energy of the excreta and of the body gain.

The gains in live weight, the digestibility of nitrogen, and the retention of nitrogen and energy were in the order of the increasing fat content of the diets; the superiority of the 5%

over the 2% fat diet with respect to the utilization of both protein and energy being much greater than the superiority of the 30% as compared with the 5% diet.

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# RELATION OF FAT TO ECONOMY OF FOOD UTILIZATION

## II. BY THE MATURE ALBINO RAT <sup>1</sup>

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In a recent investigation of the associative dynamic effects of protein, carbohydrate and fat, by Forbes and Swift ('44), an effort was made to differentiate between the individual influences of these nutrients on the heat production by determining their specific dynamic effects separately and also in the four possible combinations of these three kinds of nutriment, each such supplement being superimposed upon a complete diet sufficient for maintenance. In thus recognizing the heat production of energy equilibrium as the standard base from which to measure nutritive energy value, it is not implied, and it is not the understanding of the authors that the heat increment of nutrients retained as body increase is the same as that of nutrients catabolized; but it is their belief that after the diet has satisfied the unavoidable non-productive energy expense of maintenance, the heat production of energy equilibrium is the logical base value from which to measure the expense of utilization of food energy for whatever productive purposes it serves in the metabolic ensemble imposed by the experimental regime.

In the study just mentioned the two nutrient combinations which led to the minimum heat increments were those composed of beef protein and lard, and of cerelose (corn sugar)

<sup>1</sup> Authorized for publication on September 12, 1945, as paper no. 1288 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

and lard. Next in the increasing order of dynamic effects was the combination of beef protein, cerelose and lard; while the least efficient combination, as indicated by the maximum heat increment, was the one containing no fat, and which was composed of cerelose and beef protein. The implication, therefore, was that lard, in its combinations with cerelose and with beef protein, conferred economy of utilization of food energy, as indicated by low heat increments.

As a means of throwing further light on the influence of fat on economy of food utilization, therefore, two studies, one with growing and the other with mature rats as subjects were conducted; and the first of these experiments having been discussed (Forbes, Swift, Elliott and James, '46), an account of the second is now presented.

Since the general method of this experiment involves the determination of the specific dynamic effects of nutrients, the results of which are much affected by the details of the procedure followed, the experimental requirements of this estimation will be considered. The general principle of measurement of the dynamic effects of nutriment is to determine the heat increment resulting from the feeding of the test substance to the experimental subject in some basal status. In this relation one's first thought is inevitably of Rubner's ('02) measurement of the specific dynamic effect of protein, based upon the heat production of a fast.

It is the opinion of the authors that the specific dynamic effects of nutrients, in the sense in which Rubner used the term, are without definite significance as measures of the energy expense of nutrient utilization, for the reasons that the heat production of a fast, at the expense of body nutrients, comprises two factors, a net energy and an energy expense of utilization, exactly as does metabolizable energy of food origin; and that a part of the energy value of nutrients fed to a fasting animal is inevitably used to spare the catabolism of body nutrients. Therefore the observed increase in heat production is less than the true energy expense of utilization of the food substance.

This idea of an energy expense of utilization of body nutrients catabolized is not new; in fact it was clearly expressed in connection with one experiment by Rubner ('02, p. 371) himself. In this instance he definitely interpreted the increased heat production in phlorhizin glycosuria, in comparison with that of fast, as representing a specific dynamic effect of the body protein catabolized.

Without undertaking to review all the literature on this point, a few of the many supporting expressions may be cited. Adams's ('26) confirmation of Oppenheimer's theory that the cells of the body must do chemical work in order to bring about some of the metabolic functions following the ingestion of food, by reference to the second and third laws of thermodynamics. Another is the proposal by Williams, Riche and Lusk ('12) of a method of calculating specific dynamic effects of protein which recognizes the dynamic effect of body protein catabolized during fast. Lusk ('28) positively expressed his approval of Rubner's conclusion that there is a specific dynamic effect of catabolized body protein. Borsook and Winegarden ('31) observed a close correlation between the specific dynamic effect of protein and the increase in urinary nitrogen excretion as compared with that of the basal levels. Finally, we may cite Borsook's ('36) conclusion that there is no reason to expect different energy exchanges whether tissue protein and amino acids, or amino acids immediately derived from ingested proteins, are metabolized. The subject of dynamic effects of body nutrients has also been discussed by Forbes and Swift ('41), who presented results of an effort to measure such values.

In spite of Rubner's ('02, p. 371) conclusion that there is a dynamic effect of body nutrients catabolized, and the general approval of the principle involved, however, this conception has not been generally followed through to its logical consequence in the determination of dynamic effects of nutrients, either by Rubner or by those who have followed his lead. This logical consequence would be the recognition of the fasting catabolism minus the dynamic effect of the body nutrients

catabolized as the theoretically correct base value of heat production. An excuse, if not an adequate reason, for the general failure to recognize this point of view is that there seems to be no entirely defensible method for determining the dynamic effect of body nutrients catabolized.

It is, therefore, the understanding of the authors that determinations of dynamic effects of nutrients made with any submaintenance base value of heat production are in error, as observed, by the amount by which the metabolizable energy of the test nutrients serves to diminish the dynamic effect of the body nutrients catabolized.

In addition to this error, other reasons why the heat production at a submaintenance plane of nutrition is not a favorable one from which to measure the dynamic effects or heat increments of nutrients are (a) that nutrition at a submaintenance level is quite irksome to the experimental subject, and is therefore likely to lead to inconsistent response to the food; and (b) that the heat production at a submaintenance plane of nutrition is a difficultly measurable value, without standard significance, since its magnitude is decreased during the continuance of the submaintenance status by a rapid decrease in heat from carbohydrate, a slower decrease in heat from protein, and a decrease in heat from fat the extent of which is largely dependent on the fatness of the animal.

According to the authors' understanding the determination of the most significant measurements of the energy expense of nutrient utilization depends upon having the experimental conditions such that there are the minimum differences in the quantities of nutrients of the kinds metabolized in the two measurements of heat production compared. These conditions seem to be met in the most practicable manner when the test nutrient is superimposed upon a nutritively complete diet fed in a quantity sufficient for energy equilibrium.

Another extremely important consideration relating to the determination of the energy expense of utilization of nutrients is the method of feeding—whether the effect as observed is a short-time measurement derived from a single portion

of the test substance fed to an animal without preliminary treatment, or from a much longer-time measurement at an established plane of nutrition to which the animal has been adapted by adequate preparatory treatment.

Three reasons why heat increments should be determined by difference between results from established states of nutrition rather than from single feedings of test nutrients are the following: (a) animals are highly adaptable to conditions of nutrition, and the economy with which they utilize nutrients is much affected by the need for thrift; a heat increment representing conditions to which an animal has become adjusted, therefore, is much more reliably representative of the diet than is an increment representing a single feeding to an animal not so adjusted, (b) periods of heat measurement with animals which have been adjusted to their diet by preliminary treatment may be as long as required in the interest of accuracy, while observations based on single feedings are limited to intervals between times of feeding, (c) in the single-feeding method an accurate measurement of the heat increment is rendered impracticable by the technical difficulty of accounting for all of the increase in heat; in fact this frequently is not even attempted, conclusions being based on the highest heat production observed, or on the rate of heat production during some short time interval, the values so derived in either case standing in no particular relation to the total heat increment.

It is also favorable to consistency of heat increments that the experimental subjects be habituated to the other environmental conditions, especially as to air temperature, which are to prevail during the determination.

Furthermore, the experience of the authors suggests that there are seasonal effects to be reckoned with in research in energy metabolism, and therefore a program of experimentation running through a number of months should be so planned that the accumulation of a satisfactory volume of evidence is accomplished not by a single, inclusive experiment requiring the whole period for its execution, but by repeated ex-



periments with smaller numbers of subjects at a time, each such sub-experiment being complete in the sense of covering the entire ground of the project.

In the determination of heat increments based on the heat production of approximate energy equilibrium it is assumed that the energy expense of maintenance is unaffected by the plane of nutrition, and that this quota cancels out in the computation of the increments, the difference in heat production at the two planes of nutrition, therefore, representing the energy expense of food utilization.

#### EXPERIMENTAL

The purpose of the present experiment was to determine the energy expense of utilization (dynamic effect) of complete diets as affected by their fat contents. The experiment was conducted by the open-circuit, respiratory quotient method of Haldane, with the mature albino rat as the subject. The diets contained 2, 5, 10 and 30% of fat, respectively, and were so compounded and fed as to supply identical quantities of energy, protein, and vitamins. The composition of the diets fed in this experiment was exactly the same as that of the diets fed in the experiment reported in the preceding paper (see its table 1), except that during the last quarter of the experiment the intake of calcium pantothenate was increased five-fold, to 250 mg. per kilogram of diet no. 1, and of isocaloric quantities of the other diets.

The daily intake of gross nutrients is recorded in table 1, in which it is shown that while on either maintenance or super-maintenance feeding the mature rats on the four diets received the same quantities of energy and of protein, but different quantities of fat, and compensating differences in carbohydrate.

In table 2 is given the general schedule of experimentation. The subjects were 48 male rats constituting four groups of 12 each, one group for each diet, each of these four groups containing one rat from each of 12 litters. The rats had been reared for the experimental program at temperatures within

the zone of thermal neutrality, and were born on such dates that each individual was 205 days old when first used in the respiration chamber on the maintenance diet, and 212 days old when first used on the supermaintenance diet.

The heat production was measured for each rat for each dietary treatment during the greater part of 2 consecutive working days; heat increments being determined as the difference in heat production of the same 12 rats on maintenance

TABLE 1

*Percentage contents of diets, and daily intake of protein, carbohydrate, fat and energy.*

DIET NO.	PLANE OF NUTRITION	TOTAL FOOD IN-TAKE	PROTEIN		CARBO-HYDRATE		FAT		ENERGY
		gm	%	gm.	%	gm.	%	gm.	Cal
1	Maintenance	11.00	20.00	2.20	67.78	7.46	2.07	.23	44.64
2	Maintenance	10.52	20.90	2.20	63.68	6.70	5.07	.53	44.64
3	Maintenance	9.81	22.41	2.20	57.66	5.66	10.08	.99	44.64
4	Maintenance	7.73	28.48	2.20	32.70	2.53	30.10	2.33	44.64
1	Supermaintenance	16.00	20.00	3.20	67.78	10.84	2.07	.33	64.93
2	Supermaintenance	15.31	20.90	3.20	63.68	9.75	5.07	.78	64.93
3	Supermaintenance	14.28	22.41	3.20	57.66	8.23	10.08	1.44	64.93
4	Supermaintenance	11.25	28.48	3.20	32.70	3.68	30.10	3.39	64.93

TABLE 2

*Schedule of experimentation.*

RAT NOS.	PLANES OF NUTRITION	DAILY ENERGY INTAKE	AGE OF RATS	AVERAGE WEIGHTS OF RATS	DATES OF RESPIRATION MEASUREMENTS
		Cal.	days	gm.	
1-12	Maintenance	44.64	205	353	Jan. 29-Feb. 3
1-12	Supermaintenance	64.93	212	381	Feb. 5-10
13-24	Maintenance	44.64	205	314	Feb. 26-March 3
13-24	Supermaintenance	64.93	212	336	March 5-10
25-36	Maintenance	44.64	205	319	April 23-28
25-36	Supermaintenance	64.93	212	344	April 30-May 5
37-48	Maintenance	44.64	205	320	May 21-26
37-48	Supermaintenance	64.93	212	344	May 28-June 2

and supermaintenance quantities of the same diet. Voluntary activity during respiration experiments was excluded by subjecting the rats to a bright light, which made them keep their eyes shut and remain generally motionless.

In order to equalize possible effects of the time of year on the results, the investigation was conducted as a sequence of four identical programs each of which covered the whole ground of the study, with three rats as subjects. Therefore twelve rats in all were used with each experimental treatment.

The subjects were selected on the basis of their performance during 5 days' ad libitum consumption of the colony diet in individual cages. Then the selected individuals were fed a constant amount (16 gm.) of the colony diet, twice daily, for 5 days, to establish them, as nearly as possible, on a uniform rate of metabolism throughout the day, and to determine that they would probably eat equivalent quantities of the experimental diets when later called upon to do so. The subjects were then fed maintenance quantities of the four experimental diets for 10 days, to adapt them to this plane of metabolism: and during this interval each rat was subjected to the routine of the respiration experiment, on 2 days, by way of training for the respiration measurements to follow.

This preliminary maintenance period was followed by 8 days' feeding on the same quantities of the diets, with collection of the excreta for the determination of nitrogen balance and metabolizable energy. Then followed 2 days of respiration measurement on the maintenance diets. The subjects were next given the supermaintenance diets for 5 days to adapt their heat production to this level of food intake, the weight of the diets being 16 gm. for diet no. 1, and somewhat less for the diets of higher fat contents as required to provide isocaloric food intake. Next followed 2 days of respiration measurement, and finally an 8-day period of excreta collection, both on the supermaintenance diets.

A purpose in the order of feeding treatment, as recounted, was to have the respiration experiments on the two planes of food intake as close together as possible, that is, separated

only by the 5 days required to establish the animals in heat production representative of the higher plane of food consumption.

During the 2 days' measurements of the heat production, the rat, after receiving food at 6:30 A.M., was put into the respiration apparatus at 8:00 A.M., where it remained until 3:00 P.M.

The weight of the carbon dioxide absorbed, and the degree of physical activity of the experimental subject as indicated by a work adder, were recorded each hour from 9:00 A.M. until 3:00 P.M., and the hourly values used for heat production were selected as those which did not differ from the average for the day (excluding the first hour) by more than 6%. Incidentally this exclusion of the more aberrant data made no significant difference in the experimental findings. The weight of carbon dioxide eliminated during the entire 7-hour period was used in computing the respiratory quotient, but only the amount eliminated during the selected intervals of quiet were used in computing the heat production. It was assumed that the total respiratory quotient was unaffected by the slight activity of the animal during the 7-hour respiration period. The definite significance of the results obtained, therefore, was limited to the selected intervals of quiet, without evidence or claim that they exactly represented the entire 24-hour day. It is also conceded that there was probably a difference between the amounts of heat produced by the subjects while asleep and while awake but motionless.

The respiration experiments were conducted with the apparatus at a temperature of  $28^{\circ}\text{C.} \pm 0.1^{\circ}\text{C.}$ , the temperature inside the respiration chamber being about  $29^{\circ}\text{C.}$ , which is within the zone of thermal neutrality. When the rats were not in the respiration chambers they were in a room the temperature of which was thermostatically controlled at  $29^{\circ}\text{C.}$

The data in table 3 show that the nitrogen of the urine during the intended maintenance feeding was close to the requirement for equilibrium under the prevailing dietary conditions; and the greater amount of nitrogen in the urine

from supermaintenance than from maintenance feeding may be considered as resulting from the materially greater amount of digested nitrogen available. While it would be desirable, in determining dynamic effects of nutrients, to have the same quantity of nitrogen catabolized in both the basal and the supplemented periods, this is apparently an impossible objective. On comparing the nitrogen retention from the 2% and the 30% fat diets by these approximately mature rats it appears that the odds were only 4 to 1 that the observed

TABLE 3  
*Utilization of daily nitrogen.*

DIET NOB.	PLANE OF NUTRITION	FAT CON- TENT OF DIET	NITRO- GEN IN- TAKE	NITROGEN DIGESTED		NITROGEN OF URINE		NITRO- GEN RETEN- TION
		%	mg.	mg.	% of intake	mg	% of intake	% of intake
1	Maintenance	2	352	319	90.6	325	92.3	— 1.7
2	Maintenance	5	352	318	90.3	327	92.9	— 2.6
3	Maintenance	10	352	320	90.9	316	89.8	1.1
4	Maintenance	30	352	322	91.5	330	93.8	— 2.3
1	Supermaintenance	2	512	461	90.0	415	81.1	9.0
2	Supermaintenance	5	512	461	90.0	416	81.3	9.0
3	Supermaintenance	10	512	463	90.4	415	81.1	9.4
4	Supermaintenance	30	512	467	91.2	410	80.1	11.1

difference was significant; the odds were many millions to one that the corresponding values for energy retention were significant.

The metabolizable energy values of the daily food (table 4) were virtually constant for the four diets at the maintenance and at the supermaintenance planes of food intake, with a range of coefficients of variation from 0.3% to 0.8%.

The heat production from the daily food intake, however, at both planes of nutrition, diminished in the increasing order of the fat contents of the diets, without exception; and the coefficients of variation of the heat production (3.0% to 5.6%), while much higher than for metabolizable energy, were satisfactorily low for this observation.

The average coefficient of variation of the determined values for metabolizable energy of the four diets was 0.46%, and for the heat production from these diets it was 4.1%; and the computed odds that the average heat increment value of the 30% fat diet was less than that of the 2% fat diet were many millions to one. Of the greater amount of energy stored 67% was protein.

With respect to the plane of nutrition of the rats during the period of approximate energy equilibrium—three groups lost and one group gained slight amounts of nitrogen (table

TABLE 4  
*Partition of daily food energy.*

DIET NOB.	PLANES OF NUTRITION	INTAKE	FECES	URINE	METABOLIZABLE ENERGY		HEAT PRODUCTION	
		Cal.	Cal.	Cal.	Cal.	coeff. var. %	Cal	coeff. var. %
1	Maintenance	44.64	2.25	2.75	39.64	.3	30.50	5.6
2	Maintenance	44.64	2.35	2.75	39.54	.4	29.93	3.8
3	Maintenance	44.64	2.16	2.75	39.73	.5	29.50	3.2
4	Maintenance	44.64	2.53	2.76	39.35	.8	27.56	5.4
1	Supermaintenance	64.93	3.30	3.91	57.72	.3	37.79	3.8
2	Supermaintenance	64.93	3.27	3.92	57.74	.4	36.32	3.2
3	Supermaintenance	64.93	3.20	3.93	57.80	.5	35.28	3.0
4	Supermaintenance	64.93	3.58	3.96	57.39	.5	31.66	4.8

3); three groups gained slightly in weight, while the fourth exactly maintained its weight; and the values for heat production subtracted from those for metabolizable energy (table 4) show that all groups stored energy during the respiration experiments. Conditions during the maintenance period, therefore, are considered to have been satisfactory for the determination of the base value of heat production.

The gross energy of the daily dietary supplements was 20.29 cal.; and the daily heat increments (table 5), which diminished in the increasing order of the fat contents of the diets, were 7.29, 6.39, 5.78 and 4.10 cal., respectively, which amounted to 36, 31, 29 and 20%, respectively, of the gross energy of these supplements containing 2, 5, 10 and 30% of fat.

A logical question, however, as to the validity of this apparent effect of the fat content of the diet on the efficiency of utilization of food energy concerns the extent to which the requirements of energy production from fat and from carbohydrate, especially the vitamin requirements, were satisfied by the diets. It was intended that the vitamin intake should be liberal, and the amounts of thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, calcium pantothenate and

TABLE 5

*Quantities, increments and sources of average daily heat production.*

DIET NOS.	PLANES OF NUTRITION	TOTAL HEAT, AND HEAT INCREMENTS	NON-PROTEIN R. Q.	SOURCES OF HEAT PRODUCTION			
				Protein	Carbohydrate	Fat	Fat synthesis
		Cal.		Cal.	Cal.	Cal.	Cal.
1	Supermaintenance	37.79	1.18	11.01	25.72	0	1.06
1	Maintenance	30.50	1.09	8.63	21.47	0	0.40
1	Heat increments	7.29		2.38	4.25	0	0.66
2	Supermaintenance	36.32	1.15	11.03	24.50	0	0.79
2	Maintenance	29.93	1.05	8.68	21.02	0	0.23
2	Heat increments	6.39		2.35	3.48	0	0.56
3	Supermaintenance	35.28	1.07	11.00	23.91	0	0.37
3	Maintenance	29.50	1.00	8.36	21.10	0	0.04
3	Heat increments	5.78		2.64	2.81	0	0.33
4	Supermaintenance	31.66	0.85	10.87	10.54	10.25	0
4	Maintenance	27.56	0.82	8.76	7.57	11.23	0
4	Heat increments	4.10		2.11	2.97	— 0.98	0

niacin in the diets were the same as those characterized by Boutwell and associates ('45) as being at a high level. In the course of this experiment the authors received a suggestion to the effect that an increased pantothenic acid intake might wipe out the appearance of superior economy of utilization of fat as compared with carbohydrate.

In response to this suggestion an increase was made in the calcium pantothenate intake, and during the last quarter of

this experimental program, five times as much of this vitamin was fed as during the first three-fourths of the program. This increased intake of calcium pantothenate, however, did not alter the general trend of the results. The individual variation of the heat production of the rats, and apparent effects of the season of the year, were of such magnitude that the comparison of the results from the last three rats with the results from the previous nine afforded no significant basis for a conclusion as to the pantothenic acid requirement.

The observed results with respect to the effect of the fat content of the diets on the economy of utilization of food energy, therefore, may be considered as being in accord with the present state of knowledge of energy metabolism; but it is necessary to concede the possibility that future progress in understanding of the nutritive requirements of energy production from fat and carbohydrate could alter these findings.

In relation to the origin of the heat production, with the increasing fat and compensating decrease in the carbohydrate content of the diets, the amount of heat derived from protein was virtually unchanged; the heat of carbohydrate catabolism was decreased; there was no heat from fat catabolism except from the 30% fat diet; and there was a decreasing quantity of heat from fat synthesis, there being no heat from this source from the 30% fat diet. The sources of the decreasing energy expense of utilization of the isocaloric diets, in the order of their increasing fat contents, therefore, were decreasing heat from the catabolism of carbohydrate and from fat synthesis. In this factoring of the heat increment, the portion ascribed to fat synthesis was conventionally considered to be exclusively a product of carbohydrate catabolism, whereas, it will be understood, protein also presumably contributed to this quota.

The heat increments reported from this investigation were of a general order of magnitude similar to those derived in other recent experimental work conducted by the same procedure in this laboratory. The reason that they were much larger than those commonly reported in the literature lies



primarily in the difference in the base values from which the increments were computed. In the present study these base values of heat production were near to that of energy equilibrium, the increase in heat production incident to the consumption of the test supplements, therefore, representing the increased energy expense of food utilization; while most of the determinations of dynamic effects reported in the literature have been determined by procedures implying some sub-maintenance level of heat production as the base value, this value being too high, and the heat increments too low, to represent the food correctly, the extent of this error being that to which the heat increment as observed had been diminished by heat derived from body nutrients catabolized.

For the avoidance of confusion, the authors call attention to the facts that the results reported in the present and the foregoing paper represent growing animals during voluntary activity, and mature animals at rest, on diets comparatively low in fat content, by virtue of which they are not in conflict with the numerous published findings that work is performed more efficiently at the expense of carbohydrate than of fat.

#### SUMMARY

Respiration experiments were conducted by the open-circuit, Haldane respiratory quotient procedure, with 48 mature albino rats as subjects, to investigate the energy expense of utilization (heat increment) of complete diets as affected by their contents of fat.

Heat increments were measured as the difference in heat production from maintenance and supermaintenance diets containing 2, 5, 10 and 30% of fat, respectively, fed in such a way as to supply equal quantities of gross energy, of protein, and of vitamins.

In harmony with results of a growth experiment in which the same diets were fed, the digestibility and the retention of food nitrogen were highest when the diet containing 30% of fat was used.

The metabolizable energy of the diets was unaffected by their fat contents.

The heat production at both planes of nutrition, and also the heat increments, diminished in the order of the increasing fat contents of the diets.

The heat increments of the dietary supplements containing 2, 5, 10 and 30% of fat, respectively, were equivalent to 36, 31, 29 and 20%, respectively, of their gross energy.

The decreasing energy expense of utilization of the isocaloric diets, in the order of their increasing fat contents, was due to decreasing heat from the catabolism of carbohydrate and from fat synthesis.

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# VITAMIN A, ASCORBIC ACID AND SPINAL FLUID PRESSURE RELATIONSHIPS IN THE YOUNG BOVINE <sup>1</sup>

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In a previous publication Moore and Sykes ('40) reported that vitamin A deficiency in the young bovine was associated with an increased spinal fluid pressure. In extreme vitamin A deficiency pressures as high as 600 mm. of saline were noted by Moore and Sykes ('41), an increase of about six-fold above normal. Boyer, Phillips and co-workers ('42) suggested that the increased spinal fluid pressure noted in vitamin A deficiency was associated with a decreased synthesis of ascorbic acid. These workers produced a reduction in spinal fluid pressure by the subcutaneous injection of ascorbic acid in three out of five calves. Moore, Berry and Sykes ('43) found no particular relationship between the level of blood plasma ascorbic acid and increased spinal fluid pressure. However, the recorded pressures were not high and the variations in carotene intake were small so that the authors withheld further comment until more critical experiments were completed. These experiments have been completed and are reported herein.

## EXPERIMENTAL

Three groups of calves were used. The three calves in the first group had been receiving various quantities of carotene

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in the form of dehydrated alfalfa leaf meal in studies on carotene requirements. Calf 468 had been receiving sufficient carotene to maintain a normal spinal fluid pressure. Calves 474 and 472 had not been receiving sufficient carotene to maintain a normal spinal fluid pressure and thus showed some elevation of pressure at the time they were used in the present experiment. The carotene supplement was removed from the ration of all three calves in order to cause increases in spinal fluid pressure. One of these calves, animal 468, after developing some increased spinal fluid pressure was then injected subcutaneously with 1 gm. of ascorbic acid daily for 13 days.

The two calves used in group II were similar to those in group I. These two calves had been receiving sufficient carotene to prevent any increase in spinal fluid pressure. The carotene supplement was removed from the ration at which time 3 gm. of chlorobutanol per day were added to the ration. Since several laboratories have shown that this drug stimulates the synthesis of ascorbic acid in animals, the purpose in this instance was to determine whether or not the extra synthesized ascorbic acid would prevent the development of an increased spinal fluid pressure in the vitamin A deficient calf.

There were two calves in group III. They were about 3 months of age and had not been used previously for experimental purposes. They were placed on the vitamin A deficient ration with adequate supplementation for 30 days after which the supplement was cut down to variable amounts. The supplement was fed in just sufficient quantity to maintain fair health and appetite, but not in sufficient quantity to prevent an increase in spinal fluid pressure. Beginning at 183 days of age 1 gm. of ascorbic acid per day was injected subcutaneously. The purpose of using these calves was to maintain normal or above normal ascorbic acid values in blood and spinal fluid and to determine whether or not the vitamin A deficient calf would still develop an increased spinal fluid pressure.

Plasma vitamin A and carotene determinations were made each week by a modification of a previously published pro-

cedure for carotene by Moore ('39). Plasma ascorbic acid determinations were made by the method of Mindlin and Butler ('38). When spinal punctures were made 3 ml. of spinal fluid were withdrawn on which ascorbic determinations were made by the same procedure as used for blood plasma. Where ascorbic acid determinations were made on whole blood, the method of Roe and Keuther ('43) was used. A photoelectric spectrophotometer was used for the chemical determinations.

Spinal punctures were made by the method of Sykes and Moore ('42). The pressure was measured by connecting the puncture needle to a small bore glass tube. The height to which the spinal fluid rose in the tube plus the length of the needle was recorded as the spinal fluid pressure.

When 1 gm. of ascorbic acid was injected subcutaneously, just prior to use it was dissolved in 15 ml. of water containing 750 mg. of trisodium phosphate, which partially neutralized the ascorbic acid.

The blood and spinal fluid samples used for chemical analysis were drawn about 18 hours after the previous subcutaneous injection of ascorbic acid.

#### RESULTS AND DISCUSSION

The results with the first group of three calves are shown in table 1 which confirms in part the observations made by Boyer and coworkers ('42). Thus when the carotene supplement was removed from the ration of animal 468 there was an increase in spinal fluid pressure associated with a depression in plasma vitamin A, carotene, ascorbic acid and spinal fluid ascorbic acid values. The plasma ascorbic acid values were not depressed until after 441 days. However, the injection of 1 gm. of ascorbic acid for a period of 13 days caused no depression in spinal fluid pressure.

Practically all the plasma ascorbic values shown in table 1 for animals 472 and 474 were low even before the removal of the alfalfa meal. These two animals had not been receiving sufficient carotene to maintain a normal spinal fluid pressure so that the low plasma values even before the removal of the

TABLE 1

*Effect of vitamin A deficiency on spinal fluid pressure, plasma and spinal fluid ascorbic acid values.*

AGE	P L A S M A			S P I N A L F L U I D		
	Vitamin A	Carotene	Ascorbic acid	Ascorbic acid	Pressure	
days	μg./100 ml.		mg./100 ml.		mm. H <sub>2</sub> O	
<i>Animal 468</i>						
357	6.9	38	0.33			
371	10.8	33	0.36	3.72	95	
392	9.3	29	0.30			
406	9.3	37	0.37			
413	8.4	39		3.44	100	
alfalfa meal removed at 417 days						
420	6.3	33	0.43			
427	4.5	23	0.46			
434	3.3	17	0.38			
441	2.7	12	0.27	1.02	150	
448	0.9	12	0.20			
455	1.6	10		1.86	240	
462	1.2	8	0.27			
1 gm. ascorbic acid daily at 463 days						
469	0.3	10	0.32			
476			0.25	1.42	280	
<i>Animal 472</i>						
363	6.0	53	0.13		160	
377	10.5	40				
384	10.5	47	0.14			
398	9.6	29	0.20			
405	10.8	40		2.18	180	
alfalfa meal removed at 409 days						
412	9.0	32	0.28			
AGE	P L A S M A			S P I N A L F L U I D		
	Vitamin A	Carotene	Ascorbic acid	Ascorbic acid	Pressure	
days	μg./100 ml.		mg./100 ml.		mm. H <sub>2</sub> O	
<i>Animal 472 (continued)</i>						
419	4.8	25	0.21			
426	1.8	10	0.30			
433	0.9	6	0.26		2.42	270
440	0.9	4	0.21			
447	1.8	7	0.22		2.01	210
454	3.1	5	0.23			
461	1.8	7	0.15			
468				0.66	1.55	
<i>Animal 474</i>						
359	7.5	25	0.11	3.07	170	
380	7.2	32	0.26			
387	7.1	25				
394	9.0	34	0.24			
401	3.9	25		2.67	170	
alfalfa meal removed at 405 days						
408	6.3	22	0.33			
415	2.7	18	0.33			
439	0.3	9	0.28	1.59	300	
446	0.9	5	0.23			
453	1.8	3	0.25	1.49	310	
460		3	0.22			
467	0.3	4	0.23			
474	1.8	2	0.37		2.55	

source of carotene would be expected and in line with the observations of Boyer and coworkers ('42).

The results with the second group of two calves are shown in table 2. When the source of carotene was removed from the ration 3 gm. of chlorobutanol per day were added. In both calves the plasma vitamin A and carotene values decreased when the alfalfa meal was removed from the ration. The

chlorobutanol maintained normal ascorbic acid values in both plasma and spinal fluid. Nevertheless, the spinal fluid pressure increased. When the chlorobutanol was discontinued and 1 gm. of ascorbic acid per day was injected subcutaneously for 10 days there was no decrease in spinal fluid pressure.

TABLE 2

*Effect of feeding chlorobutanol on spinal fluid pressure, plasma and spinal fluid ascorbic acid values in vitamin A deficiency.*

AGE	P L A S M A			SPINAL FLUID		
	Vitamin A	Carotene	Ascorbic acid	Ascorbic acid	Pressure	
days	µg./100 ml.	mg./100 ml.			mm. H <sub>2</sub> O	
<i>Animal 466</i>						
442	11.7	65				
449	10.8	64		3.32	90	
456	11.7	59				
463	10.2	59	0.36			
484	10.2	58	0.31			
491	13.2	55		2.81	80	
alfalfa meal removed at 499 days						
498	10.8	68	0.49			
3 gm. chlorobutanol daily at 503 days						
505	9.0	38	0.59			
512	5.7	31	0.44			
519	3.3	18	0.28	3.33	190	
526	3.3	17	0.34			
533	3.0	12	0.29		200	
540	3.6	14	0.39			
chlorobutanol discontinued						
1 gm. ascorbic acid daily at 544 days						
547	2.1	21	0.51			
554	3.0	19	0.21		300	
<i>Animal 467</i>						
403	7.8	71	0.28	2.40	100	
410	11.4	67				
417	10.2	61				
424	10.2	62	0.31			
431	10.8	73				
445	12.9	73		2.26	105	
alfalfa meal removed at 449 days						
452	12.0	59	0.34			
3 gm. chlorobutanol daily at 454 days						
459	6.3	28	0.38			
466	6.3	22	0.28			
473	4.2	14		2.15	180	
480	2.4	12	0.50			
487	3.3	10	0.44	2.30	210	
494	2.1	5	0.46			
501	0.9	9	0.48			
508		7	0.33	2.51	250	

The results of injecting ascorbic acid for a long period of time into calves on subminimal quantities of carotene are shown in table 3. As a result of the low level of carotene intake the plasma vitamin A and carotene values were depressed. As a result of injecting 1 gm. of ascorbic acid per day the ascorbic acid values for plasma, whole blood and spinal



fluid were maintained at a normal level. However, the spinal fluid pressure increased.

The results obtained with the three groups of calves show that ascorbic acid is not involved in the maintenance of a normal spinal fluid pressure in vitamin A deficient calves as indicated by Boyer and co-workers ('42). When normal blood and spinal fluid ascorbic acid values were maintained by the feeding of chlorobutanol or the subcutaneous injection of ascorbic acid the spinal fluid pressure increased as the vitamin

TABLE 3

*Effect of subcutaneous injection of ascorbic acid on spinal fluid pressure, plasma and spinal ascorbic acid values in vitamin A deficiency.*

AGE	P L A S M A			W H O L E B L O O D S P I N A L F L U I D			AGE	P L A S M A			W H O L E B L O O D S P I N A L F L U I D		
	Vitamin A	Carotene	Ascorbic acid	Ascorbic acid	Ascorbic acid	Pressure		Vitamin A	Carotene	Ascorbic acid	Ascorbic acid	Ascorbic acid	Pressure
days	μg./100 ml.	mg. per 100 ml.	mm. H <sub>2</sub> O				days	μg./100 ml.	mg. per 100 ml.	mm. H <sub>2</sub> O			
<i>Animal 88</i>							<i>Animal 87</i>						
109	12.0	36	0.39	0.83	.	.	109	12.6	56	0.39	1.02	.	.
116	11.4	26	0.47	0.67	.	.	116	15.0	61	.	0.67	.	.
123	21.0	58	0.45	0.77	.	.	123	19.2	76	0.47	1.07	.	.
130	13.1	34	0.42	0.87	.	.	130	16.5	76	0.55	1.06	.	.
137	13.1	16	0.44	0.77	.	.	137	14.1	51	0.44	0.77	.	.
144	5.7	10	0.30	0.72	.	.	144	9.9	20	0.50	0.93	.	.
151	3.3	5	0.41	0.61	.	.	151	9.0	7	0.55	0.63	.	.
166	5.7	15	0.46	0.71	3.12	60	166	9.6	16	0.54	0.81	2.43	60
179	7.5	16	0.23	0.31	.	.	179	6.0	15	.	0.68	.	.
1 gm. ascorbic acid per day at 181 days							1 gm. ascorbic acid per day at 181 days						
186	.	0.53	0.85	3.45	90	.	186	.	0.44	0.79	3.18	90	.
200	2.1	7	0.37	0.98	.	.	200	2.1	6	0.37	1.01	.	.
207	0.9	2	0.36	0.62	3.07	120	207	2.7	4	0.61	0.95	3.34	90
214	1.2	3	0.51	0.91	.	.	214	2.4	5	0.53	1.01	.	.
221	4.2	7	0.44	0.86	.	.	221	3.9	8	0.44	0.87	.	.
228	.	5	0.45	0.78	3.32	200	228	1.5	6	0.46	1.02	.	.
235	1.2	2	0.44	0.70	.	.	235	0.6	4	0.31	0.96	3.37	160
242	3.9	7	0.47	0.83	3.24	220	242	3.9	5	0.51	0.90	.	.
249	3.6	10	0.54	0.70	.	.	249	3.9	5	0.45	0.70	.	.
256	3.9	8	0.56	0.93	.	.	256	.	.	0.43	0.83	.	.
263	2.1	5	0.37	0.71	3.01	230	263	3.0	3	0.30	0.90	2.88	180
270	1.8	5	0.57	0.76	.	.	270	2.4	3	0.55	0.80	.	.

A deficiency progressed. Therefore, some other explanation for the mechanism of the increase in spinal fluid pressure in vitamin A deficiency in calves must be sought.

Boyer and co-workers ('42) obtained a depression of spinal fluid pressure in three out of five calves by the subcutaneous injection of ascorbic acid. However, they make one very significant statement. "Administration of ascorbic acid for periods longer than 1 week was impractical because of the moribund condition of the calves." It should be noted that Moore and Sykes ('41) state in a paper prior to the one published by Boyer and co-workers ('42), "In two cases there was a terminal drop in pressure from a previously high level. Usually the animals in this condition had very little appetite, showed diarrhea and were more or less in a moribund state so that the drop was not surprising." Apparently the Wisconsin workers injected ascorbic acid at a time when the calves naturally showed a drop in spinal fluid pressure because of their moribund condition.

Boyer, Phillips and co-workers ('42) further suggest that ascorbic acid may have some direct bearing on bone anomalies characteristic of vitamin A deficiency. It is of considerable interest that after 12 months on a very low carotene intake the two calves considered in the present report which have received daily subcutaneous injections of ascorbic acid have gone blind due to constriction of the optic nerve as reported by Moore, Huffman and Duncan ('35).

#### SUMMARY AND CONCLUSIONS

1. Calves rendered severely vitamin A deficient showed an increased spinal fluid pressure accompanied by depressed ascorbic acid values in blood and spinal fluid.

2. However, when normal ascorbic values in blood and spinal fluid were maintained by the feeding of chlorobutanol or the subcutaneous injection of ascorbic acid there was still an increase in spinal fluid pressure in the vitamin A deficient calves.

3. It must be concluded that the disturbance in the synthesis of ascorbic acid in the vitamin A deficient calf plays no part in the mechanism of the increased spinal fluid pressure in calves.

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# THE EVALUATION OF PROTEINS IN HYPOPROTEINEMIC DOGS<sup>1</sup>

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## THREE FIGURES

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The relation between nitrogen balance and absorbed nitrogen in normal adult dogs was described by Allison and Anderson ('45) in the following equation:

$$NB = K (AN) - NE_0 \quad (1)$$

where NB is nitrogen balance, AN absorbed nitrogen, and  $NE_0$ , the excretion of nitrogen on a protein-free diet. The value of K was constant in the region of negative nitrogen balance but it became a decreasing variable well on the positive side. This value of K was described as a measure of the rate of change of nitrogen balance with respect to absorbed nitrogen, an index to the utilization of a protein by the dog. If  $NE_0$  is the so-called "endogenous" nitrogen then K becomes the biological value defined by Thomas and elaborated by Mitchell and co-workers (Mitchell, '44). Possibly K can be defined best as a nitrogen balance index. Seeley ('45) obtained data in hypoproteinemic dogs which suggested that this index may be greater in depleted than in normal dogs. His work revealed also, that the amount of regeneration of plasma protein was a function of the magnitude of the nitrogen balance produced.

<sup>1</sup> These studies were accomplished through the support and cooperation of Sharp and Dohme, Inc., E. R. Squibb and Sons, Arlington Chemical Company, Swift and Company, and Warner Institute for Therapeutic Research.

The following work was done on hypoproteinemic dogs to determine whether equation (1) describes nitrogen balance data obtained on depleted animals and if so whether depletion affects the value of  $K$ . Further information was sought also on the correlation between the regeneration of plasma proteins and nitrogen balance.

#### METHODS

The dogs were depleted in proteins by feeding a protein-free diet (Allison and Anderson, '45) the process of depletion being speeded by plasmapheresis (Seeley, '45). Depletion resulted in a decrease in plasma albumin which was reflected by a decrease in total plasma protein (Chow, Allison, Cole, and Seeley, '45). The degree of depletion was estimated, therefore, by the magnitude of the decrease in total plasma protein which was calculated from nitrogen determinations using the micro Kjeldahl method. Previous studies proved that plasma proteins varied from 5.6 to 7.1, averaging 6.2 gm.% in normal dogs (Allison, Dreskin, and Morris, '41).

The nitrogen balance studies were conducted similarly to those on normal dogs (Allison and Anderson, '45). Protein-free feeding periods usually preceded and followed a period of nitrogen feeding. These periods lasted 4 days during which time the daily feces and urine samples were collected and pooled for analysis. Nitrogen was determined in the urine and in the feces by the micro Kjeldahl method.

The technique used to study the regeneration of plasma proteins was the same as that described by Seeley ('45). Briefly, it was this. The dogs were depleted by feeding the protein-free diet and by a few days of plasmapheresis until the plasma protein concentration was between 4.0 and 4.5 gm. per 100 ml. plasma and essentially constant under the experimental conditions used. The depleted animals were fed protein nitrogen for 5 days and then returned to the protein-free diet until the plasma protein concentration had returned to the former depleted level. Plasma protein in grams per cent was plotted against days and the area under the curves show-

ing the increase above the depleted concentration was taken as a measure of the amount of regeneration.

### RESULTS

The data in figure 1 illustrate the relationship found between nitrogen balance and absorbed nitrogen in normal and hypoproteinemic dogs fed casein. The first line represents average data previously published (Allison and Anderson, '45) obtained on normal dogs. Each of the other curves represents data obtained on single animals which had undergone protein depletion so that the plasma proteins had been reduced to 4.6, 4.6, 4.1, and 4.1 gm. per 100 ml., respectively. The value for nitrogen balance at zero absorbed nitrogen ( $NE_0$  in equation 1) varied for each dog around a mean during the series of experiments performed to establish the curve. Thus each curve was constructed by calculating all points to the average  $NE_0$ . A straight line can be drawn which will include all these points satisfactorily. Equation (1) therefore, can be applied as a linear relationship to data obtained on hypoproteinemic as well as on normal dogs in negative or low positive nitrogen balance.

Values from these same data for plasma protein concentration, absorbed nitrogen at equilibrium, nitrogen excretion on a protein-free diet ( $NE_0$ ) and the slope of the line ( $K$  in equation 1) are recorded under casein in table 1. These data prove that the amount of nitrogen necessary to maintain nitrogen equilibrium (absorbed nitrogen at equilibrium) in the depleted is less than in the normal animal. Similarly  $NE_0$  is reduced in the hypoproteinemic dog. The reduction in the amount of nitrogen necessary to maintain equilibrium can be attributed in part, therefore, to reduced protein stores as reflected by the reduction in  $NE_0$ . But the value for  $K$  (biological value or nitrogen balance index) increases from an average of 0.81 in the normal to 0.93 in the most depleted dogs. Thus, insofar as  $K$  measures retention of nitrogen, casein is utilized better in the depleted animals, another reason for the decrease in absorbed nitrogen at equilibrium.

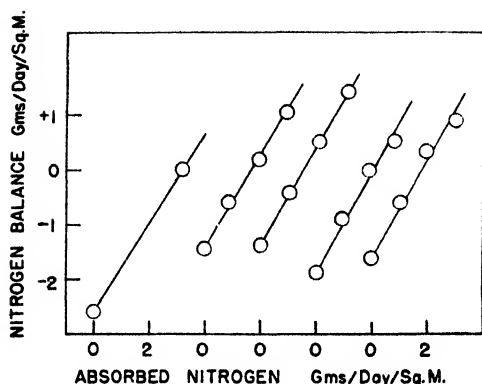


Fig. 1 Absorbed nitrogen gm./day/sq.M. plotted against nitrogen balance gm./day/sq.M. The first line represents average data obtained on normal dogs (Allison and Anderson, '45). The other four curves represent data obtained on individual hypoproteinemic dogs with plasma protein concentrations of 4.6, 4.6, 4.1 and 4.1 gm. per cent, respectively.

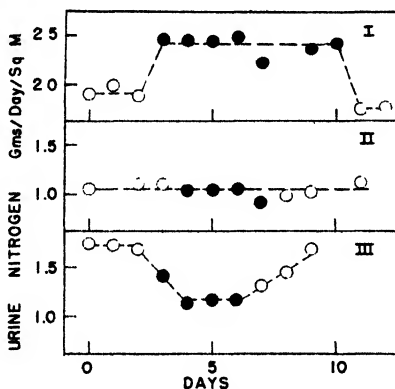


Fig. 2 Urine nitrogen gm./day/sq.M. plotted against time in days. The open circles represent data obtained while feeding protein-free diet, the solid circles data obtained while feeding protein nitrogen. See text for further description.

The following derivation illustrates the significance of  $K'$  and "net protein" value headings for the last two columns in table 1. Absorbed nitrogen (AN) was calculated from ingested nitrogen (NI), using the so-called true digestibility (D) as follows:

$$AN = NI \times D \quad (2)$$

Equation (1) can be rewritten therefore, as,

$$NB = (K) (D) (NI) - NE_0 \quad (3)$$

Digestibility has proven to be constant within experimental error so that,

$$NB = K' (NI) - NE_0 \quad (4)$$

TABLE 1

*The effect of protein depletion, reflected in a decrease from normal in plasma protein concentration, upon the evaluation of proteins or hydrolysates.*

DOG NO.	PLASMA PROTEIN	ABSORBED NITROGEN AT EQUILIBRIUM	NITROGEN EXCRETION ON PROTEIN-FREE DIET $NE_0$	K	K'	NET PROTEIN
	gm./100 ml	gm./day/ sq M.	gm./day/ sq M.			
Casein						
<sup>1</sup>	6.2	3.20	2.59	0.81	0.77	0.59
31	5.6	2.55	2.00	0.79	0.70	0.54
44	4.6	1.65	1.47	0.89	0.83	0.63
42	4.6	1.55	1.39	0.90	0.88	0.67
55	4.1	2.06	1.90	0.92	0.92	0.69
28	4.1	1.73	1.61	0.93	0.93	0.71
Casein hydrolysate						
44	6.0	2.15	1.72	0.80	0.76	0.59
48	5.7	2.94	2.32	0.79	0.79	0.61
31	5.0	1.49	1.39	0.93	0.90	0.70
Commercial hydrolysate B						
44	6.0	2.15	1.72	0.80	0.76	0.59
48	5.2	2.72	2.28	0.84	0.84	0.65
31	4.8	1.90	1.77	0.93	0.93	0.72
Commercial hydrolysate C						
60	6.0	4.60	2.20	0.48	0.40	0.18
48	5.0	3.50	2.10	0.60	0.52	0.23
55	3.9	2.42	1.69	0.70	0.60	0.26
Protein A						
<sup>1</sup>	6.2	6.60	2.50	0.39	0.32	0.28
50	4.4	2.90	1.65	0.57	0.51	0.45
42	4.0	1.50	1.10	0.73	0.66	0.58

<sup>1</sup> Data previously published (Allison and Anderson, '45).



where  $K'$  is the rate of change of nitrogen balance with respect to NI and is proportional to  $K$  in equation (1) through digestibility.  $K'$  increases even as does  $K$  with a tendency for the former value to approach  $K$  in the depleted animal suggesting that the factors involved in the calculation of digestibility are also altered by depletion. By multiplying  $K'$  by the fraction of protein in the source of dietary nitrogen, the "net protein" value is obtained. This value proposed by Mitchell ('44) is a measure of the efficiency of the source to furnish protein nitrogen to the animal.

Data similar to those obtained for casein are recorded in table 1 for a casein hydrolysate,<sup>2</sup> for commercial hydrolysates B<sup>3</sup> and C,<sup>4</sup> and for a protein A derived from soybean. These data support the conclusions drawn from the casein data that the amount of nitrogen necessary to maintain the depleted dog is less than in the normal dog, a change which is reflected in a tendency for  $NE_0$  to be reduced and for  $K$  or  $K'$  in equations (1) and (4), respectively, to increase. These data prove also that casein, the casein hydrolysate and commercial hydrolysate B are identical in value as measured by  $K$  or  $K'$  (values which may be called nitrogen balance indexes of absorbed and of total dietary nitrogen, respectively).

The data in table 2 on dried egg white<sup>5</sup> (uncooked) have been separated from those in table 1 and given in somewhat more detail to emphasize results not observed with other proteins. The data on egg white fed to hypoproteinemic dogs are similar to the others in that absorbed nitrogen at equilibrium and  $NE_0$  are decreased while  $K$  and  $K'$  and "net protein" value are increased above control values. There are however, outstanding differences. The value of  $K$  (0.96) which is higher than values obtained for the other proteins in normal dogs becomes greater than unity in the depleted animals. The data

<sup>1</sup> The casein hydrolysate was supplied by Dr. Bacon F. Chow of the Squibb Institute for Medical Research.

<sup>2</sup> Amigen (batch 10067) furnished by Dr. Warner M. Cox, Jr., of Mead Johnson and Company.

<sup>3</sup> Hydrolysate C was a commercially prepared hydrolysate.

<sup>5</sup> Manufactured by Swift and Company. This dried egg white is uncooked.

on dogs 54, 50, and 31 in table 2 are arranged chronologically, each determination of  $NE_0$  and NB requiring about 8 days, so that the entire experiment on each dog lasted approximately 1 month. These data prove that values for K can be greater than unity in the depleted dog. Furthermore, continued feeding of egg white results in a reduction in the value of  $NE_0$  from approximately 1.8 to 1.45 gm./day/sq.M. accompanied by a drop in K from values greater than one to approximately unity.

TABLE 2

*The effect of protein depletion, reflected in a decrease from normal in plasma protein concentration, upon the evaluation of egg white.<sup>1</sup>*

DOG NO.	PLASMA PROTEIN	ABSORBED NITROGEN AN	NITROGEN BALANCE NB	NITROGEN EXCRETION ON PROTEIN-FREE DIET $NE_0$	K	K'	NET PROTEIN
	gm./100 ml.	gm./day/sq.M.	gm./day/sq.M.	gm./day/sq.M.			
2	6.0	2.50	0	2.55	0.96	0.91	0.63
54	5.4	1.87	+ 0.19	1.81	1.07	1.05	0.76
	5.0	0.89	- 0.48	1.63	1.29	1.25	0.91
	5.0	1.44	+ 0.01	1.47	1.03	1.03	0.75
50	4.7	1.12	- 0.56	1.91	1.20	1.16	0.84
	4.9	0.84	- 0.58	1.49	1.08	1.08	0.78
	4.9	1.44	- 0.04	1.45	0.99	0.99	0.72
31	4.9	1.63	+ 0.30	1.77	1.27	1.27	0.92
	4.7	0.85	- 0.37	1.79	1.67	1.67	1.19
	4.7	1.26	- 0.17	1.46	1.02	1.02	0.75

<sup>1</sup> A dried uncooked product made by Swift and Company.

<sup>2</sup> Average data obtained on two normal dogs.

The fraction of absorbed nitrogen retained in the body of the animal (BV) is defined strictly by the following equation.

$$NB = (BV) (AN) - EN \quad (5)$$

where EN is the fraction of nitrogen excreted which can be attributed to body nitrogen ("endogenous"). For any given value for NB, equations (1) and (5) can be solved simultaneously to give:

$$EN = NE_0 - AN (K - BV) \quad (6)$$

Equation (6) proves that if  $K$  in equation (1) is greater than  $BV$ , then  $EN$  becomes less than  $NE_0$ . In this way egg white can conserve body nitrogen. Similarly conservation of body nitrogen by egg white in rats has been reported by Willman, Swanson, Stewart, Stevenson, and Brush ('45). Fractions of egg white are being prepared to try to isolate the amino acids or other substances which produce this interesting effect.

Correlations between values of  $K$  and the excretion of nitrogen in the urine are illustrated by the data in figure 2. Daily excretion of urine nitrogen in gm./day/sq.M. are plotted in this figure, first when the animals were on a protein-free diet (open circles) followed by nitrogen feeding (solid circles) and then back on the protein-free diet (open circles). Curve I was obtained on a normal dog which received 1.84 gm./day/sq.M. of nitrogen in the casein hydrolysate<sup>2</sup> during an 8-day feeding period.  $K$  was less than unity for this hydrolysate, the excretion of nitrogen in the urine increasing, therefore, during the nitrogen feeding period above that found for the protein-free period. Curve II was obtained on a partially depleted dog receiving 1.44 gm./day/sq.M. of egg white nitrogen where the  $K$  value was very close to unity. When  $K$  is unity the excretion of urine nitrogen is independent of the quantity of food nitrogen fed. Curve III represents data obtained on a hypoproteinemic dog receiving 1.52 gm./day/sq.M. of egg white nitrogen. The  $K$  value being greater than unity, the urine nitrogen excreted during the nitrogen feeding period is less than during the protein-free feeding period.

The relationships expressed by equation (1) and the others suggests that regeneration of body proteins would be most marked when the animal is in positive nitrogen balance. The data in figure 3 support this suggestion. Here plasma protein regeneration areas ( $\Delta$  plasma protein times day) are plotted against positive nitrogen balances produced. Regeneration areas,<sup>6</sup> described by Seeley ('45) and explained under methods in this paper are used to measure the effect of dietary pro-

<sup>6</sup> Some of these data were derived from Seeley ('45).

teins on regeneration of plasma proteins in the depleted animal. The circles with the crosses in figure 3 illustrate data obtained by feeding bovine serum protein, the lowest point being obtained with 1.6 gm. of nitrogen/day/sq.M. of body surface, the second lowest with 3.4 gm. and the two higher points with 6.6 gm./day/sq.M. The circles with horizontal lines, rectangles, square, and open circles illustrate data obtained by feeding 6.6 gm. of nitrogen/day/sq.M.

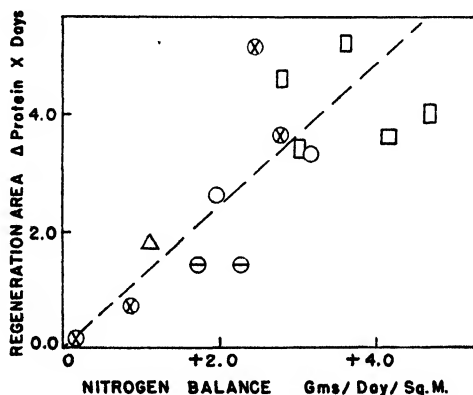


Fig. 3 Regeneration areas ( $\Delta$  protein times days) are plotted against positive nitrogen balances in gm./day/sq.M. The data was obtained on depleted dogs orally fed bovine serum protein  $\otimes$ , casein  $\circ$ , casein hydrolysate  $\square$ , hydrolysate B  $\square$  and hydrolysate C  $\ominus$ , respectively. The  $\Delta$  represents data obtained on a depleted dog fed hydrolysate B intravenously.

of commercial hydrolysate C,<sup>4</sup> casein hydrolysate,<sup>2</sup> commercial hydrolysate B,<sup>3</sup> and casein, respectively. The triangle describes one experiment when hydrolysate B was given intravenously at the rate of 8.0 gm./day/sq.M. These data prove that under the experimental conditions used, regeneration of plasma proteins increases regularly from practically zero near nitrogen equilibrium to high values when the dogs are kept well on the positive side of nitrogen balance. The amount of regeneration is a function of the nitrogen balance produced, and therefore of K and K'. These results support the conclusion of Melnick, Cowgill and Burack

('36), that there is a correlation between the nitrogen balance produced and the amount of plasma protein regeneration. The kind of plasma protein regenerated, however, will depend upon the state of depletion of the animal and the pattern of amino acids fed (Seeley, '45).

#### SUMMARY

The relationships between nitrogen balance and absorbed nitrogen and ingested nitrogen in normal and hypoproteinemic dogs are described by the equations:

$$NB = K (AN) - NE_e$$

$$NB = K' (NI) - NE_e$$

where NB is nitrogen balance, AN is absorbed nitrogen, NI is ingested nitrogen, and  $NE_e$  is the excretion of nitrogen on a protein-free diet. These are linear relationships, within experimental error, in the regions of negative and low positive nitrogen balance. K and K' are functions of the retention of nitrogen by the animal and may be called biological values or nitrogen balance indexes of absorbed and ingested nitrogen respectively.

The values for K, K' and "net protein" value increase, while those for absorbed nitrogen at equilibrium and  $NE_e$  decrease from normal in the hypoproteinemic dog.

These constants are functions of, but not necessarily the fractions of, nitrogen retained in the body of the animal since values greater than unity have been obtained while feeding egg white to hypoproteinemic dogs. Continued feeding of egg white results in a decrease in the excretion of nitrogen on a protein-free diet ( $NE_e$ ). The data can be interpreted to mean that egg white spares body nitrogen.

Regeneration of plasma protein increases in magnitude as the nitrogen balance increases on the positive side. Thus regeneration of tissue proteins in hypoproteinemic dogs as reflected in the increase of plasma proteins is a function of the nitrogen balance produced and therefore of K or K'.

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# THE EFFECT OF CHANGES IN DIET ON THE VOLUME AND COMPOSITION OF RAT MILK<sup>1</sup>

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## FOUR FIGURES

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The availability of a method to obtain milk from rats (Cox and Mueller, '37) permits a new approach to studies on lactation. The present report deals with the variations in the composition of milk from rats on a standard diet, and the effect on yield and composition induced by variations in the protein, yeast and calcium and phosphorus levels. The composition of cow's milk must be related to the fact that the cow is a herbivore and that the major constituents of her diet are hay and grass. Changes in the diet of the cow are effected by change in the type of feed which may itself introduce changes other than the single constituent under observation. Thus, for example, two hays of different calcium content may be used in obtaining different calcium levels, and at the same time the proteins of the two hays may be of different nutritional value. Such difficulties are avoided when the rat is used as the experimental animal.

## EXPERIMENTAL PROCEDURE AND RESULTS

*Stock rats.* Thirty mature stock rats were milked once, 18–20 days after parturition and the samples were each ana-

<sup>1</sup> Presented at the Memphis meeting of the American Society of Biological Chemists, April 21–24, 1937.



lyzed for fat and total protein. Eighteen additional rats were similarly milked and calcium, phosphorus and magnesium determined on each sample. The data are summarized in table 1.

The range of values for each constituent seems quite wide — but it is of the order commonly observed in surveys of the composition of cow's milk (Tocher, '25). Milk from individual cows varies widely in composition and even the pooled milk from twenty-four cows may vary as much as 1.0% in butter fat (H.M.S.O., '35). The coefficients of variation given (table 1) indicate that the protein, ash and magnesium content of rats' milk are subject to the least fluctuation and, as might be

TABLE 1

*Variations in the composition of milk from individual stock rats.*

	NO. RATS	MEAN	RANGE	STANDARD DEVIATION	STANDARD ERROR <sup>1</sup>	COEF- FICIENT OF VARI- ATION
Volume (ml./rat)	30	3.2	1.0 - 5.9	1.4	± 0.26	43.7
Fat %	30	13.3	7.1 - 19.6	3.25	± 0.59	24.4
Protein %						
(N × 6.25)	30	10.4	8.4 - 14.8	1.41	± 0.26	13.5
Ash %	18	1.43	1.15 - 1.83	0.165	± 0.040	11.5
Calcium %	18	0.325	0.242- 0.447	0.0585	± 0.014	18.0
Phosphorus %	18	0.232	0.175- 0.311	0.0395	± 0.0096	17.0
Magnesium %	18	0.025	0.021- 0.032	0.0028	± 0.00068	11.2

<sup>1</sup>  $\frac{\sigma}{\sqrt{N}}$  used for first 3 rows;  $\frac{\sigma}{\sqrt{N-1}}$  for last four.

expected, the widest variation was in the amount of milk obtained. There is some indication that the percentage protein varies with the volume of milk. The correlation coefficient was - 0.52, which is 5.8 times its probable error. This is similar to findings on cow's milk (Tocher, '25).

In the course of work to be described later, analyses for three milk constituents for two successive milkings from the same rat were made (18th and 20th day of lactation). An opportunity was thus available for determining whether the day to day variation in the composition of the milk from a single rat was equal to the differences observed between single

samples from individual rats. The fact that the rats so studied were on various diets does not detract from the validity of the differences between two values on the same individual. The standard deviations for the per cent fat, protein, and ash in rat milk (from table 1) are recorded in table 2 in order that they may be compared with the standard deviation in fat, protein and ash content of pairs of determinations on single rats. To calculate this value, the usual formula  $\sqrt{\frac{\sum d^2}{N}}$  was applied to the plus or minus variation of the second from the first determination. In this way the first of each pair of observations served as the base.

TABLE 2

*Variations in the composition of milk obtained on successive milkings.*

CONSTITUENT	RATS MILKED ONLY ONCE		RATS MILKED ON 18TH AND 20TH DAYS	
	No obs	Standard deviation	No obs.	Standard deviation <sup>1</sup>
Fat	30	3.25	28	2.70
Protein	30	1.41	35	1.46
Ash	18	0.165	29	0.199

<sup>1</sup> Calculated by algebraically subtracting the second determination of each pair of observations from the first and determining  $\sigma$ .

The data indicate that the day to day variation in the composition of milk is as great as the variation when single samples are obtained. It is impossible to obtain an entire 24-hour milk sample from rats and thus a strict comparison with cow's milk cannot be made.

*Effect of dietary protein.* Ten young mother rats were placed at parturition on each of six diets which differed only in protein (casein) content. Levels of 5, 10, 20, 30 and 50% casein were incorporated in diet no. 1 of Cox and Imboden ('36). The mother rats were milked on the eighteenth and twentieth days of lactation and as many analyses (for protein, fat and ash) run as was consistent with the volume of milk obtained.

The average total protein content ( $N. \times 6.25$ ) of the milk from the five groups was 9.8<sup>5</sup>, 9.6<sup>13</sup>, 9.7<sup>15</sup>, 10.0<sup>12</sup>; and 10.7<sup>16</sup>, respectively. (The prime figures indicate the number of determinations.) While there seems to be a small increase in the protein content with increasing intake, the differences are small and not statistically significant. This is in agreement with the general findings of Perkins ('32) on cows and others (Meigs, '22). Adair ('25) observed that human mothers on a high protein diet produced more milk than did mothers on a high fat or high carbohydrate diet. No analyses of the milk were reported.

Other constituents of the milk were somewhat modified by the protein intake. The per cent ash, per cent fat, the average volume of milk and the number and weight of young successfully raised to 21 days of age are given in figure 1. With the exception of the difference in the per cent milk fat from rats on the 20- and 30%-level, the differences in this constituent are statistically significant, the significance ratio  $\frac{M_1 - M_2}{\sqrt{P.E.^2_{M_1} + P.E.^2_{M_2}}}$  falling between 3.9 and 4.9. The variations in ash content follow the trend of fat, but the differences for the former are not significant.

As indicated in the curve, the volume of milk at low protein levels is greatly reduced, and this necessarily limits the growth and weight of young. It is interesting, however, that the average number of young raised by mothers on low protein diets was only slightly less than the number raised on diets higher in protein, although their weight was markedly reduced.

*Calcium and phosphorus.* One of the authors (Cox) and Imboden ('36) studied the effect of different levels and ratios of calcium and phosphorus on reproductive success. These studies were continued through the entire reproductive life of the rats (ten pregnancy cycles) and cannot be strictly compared with the observations below because a rat may raise one or more litters on a diet inadequate in calcium and phosphorus with fair success, so that its inadequacy may be fully

demonstrated only in later life. It was believed of interest, however, to determine the degree to which the composition of milk could be influenced by the more severe rations employed in the earlier work. It was hoped that further information on the tolerance for high phosphorus rations at low calcium intakes might be obtained. The rations employed are given in table 3.

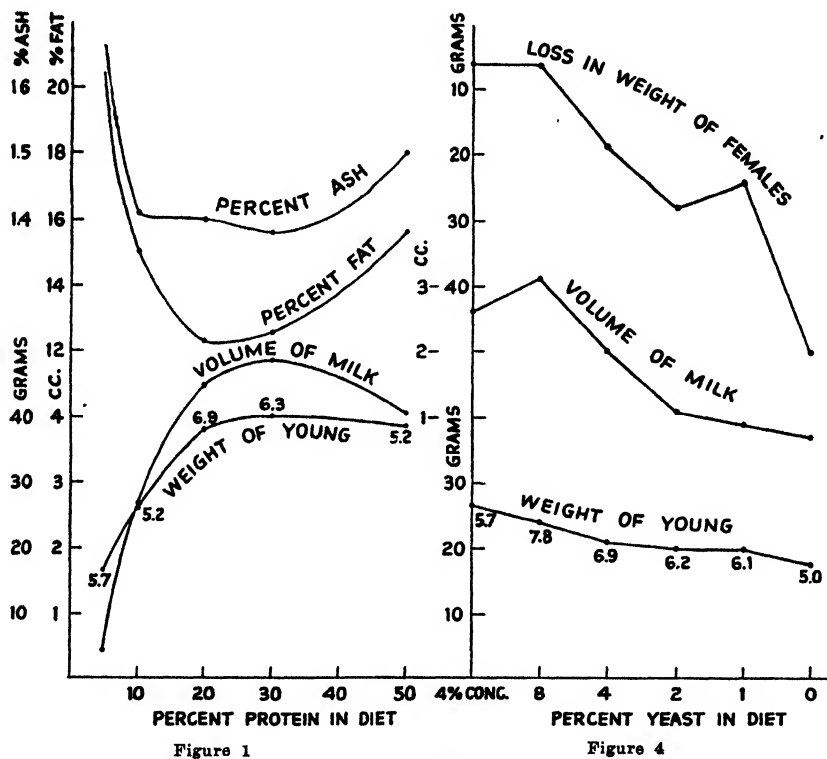


Fig. 1 The effect of the protein level of the maternal diet on the volume of milk obtained under standard conditions, the weight and number of young raised to 21 days, the ash and percentage fat of the milk. The values on the curve indicate the average number of young raised to 21 days. Ten rats were used on each diet.

Fig. 4 The effect of reducing the yeast intake of lactating mother rats as measured by their loss in weight, volume of milk and weight and number of young. Yeast or yeast concentrate was the only source of B-vitamins in the diet. The values on the curve indicate the average number of young raised by the ten rats on each diet.

The experimental procedure was exactly the same as that employed in the studies just described, save that all diets contained 20% protein. The data are presented graphically in figures 2 and 3 and confirm the earlier finding that diets with high Ca/P ratios are not as well tolerated as diets equally unbalanced as regards phosphorus. On the high calcium diets (right hand portion of the curves) the mothers lost a great deal of weight in an effort to raise their young — even though the offspring were definitely subnormal in weight and appearance. On the other hand, the high phosphorus diets did not deleteriously affect the weight of the mothers, and young of

TABLE 3  
*Levels and ratios of calcium and phosphorus employed.*

DIET NO.	CALCIUM	PHOSPHORUS	CA/P RATIO	"SUCCESS RATING" <sup>1</sup>	DIET NO.	CALCIUM	PHOSPHORUS	CA/P RATIO	"SUCCESS RATING" <sup>1</sup>
	%	%				%	%		
1	0.245	0.245	1.0	76.0	6	0.490	0.245	2.0	90.4
2	0.245	0.490	0.5	79.9	11	0.735	0.245	3.0	72.8
3	0.245	0.735	0.33	93.6	16	1.225	0.245	5.0	13.2
4	0.245	1.225	0.2	83.5	21	2.450	0.245	10.0	0.5
5	0.245	2.450	0.1	10.3	Stock	0.45	0.47	1.1	89.0

<sup>1</sup> On 10 reproductive cycles (see table 2, Cox and Imboden, '36).

good average size resulted. The difference in number of young raised on the high phosphorus diets may be fortuitous because more young were raised on diets with a P/Ca ratio of 10 than on diets with P/Ca ratios of 3. The largest volume of milk was obtained at a Ca/P ratio of 2.0, but ratios higher than this resulted in rapid decline in milk production. This was not the case with diets unbalanced in terms of phosphorus.

The composition of the milk was influenced only slightly, as indicated in figure 3. High phosphorus diets had practically no effect on the Ca, P, total ash or Ca/P ratio of the milk. With increase in dietary Ca/P ratio, however, there was progressive decrease in total ash, calcium and phosphorus. In this portion of the curve the Ca/P ratio of the milk increased with the Ca/P ratio of the diet, simultaneously with

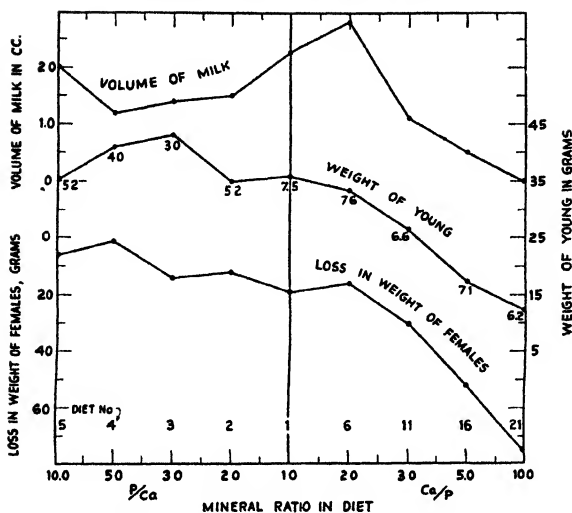


Fig. 2 The effect of various calcium and phosphorus levels in the maternal diet on the volume milk, the weight and number of young raised to 21 days of age, and the change in weight of the mother rats during lactation. High phosphorus diets are on the left, and high calcium diets on the right of the center line. At a Ca/P ratio of 1.0, the diet contained 0.245% calcium and 0.245% phosphorus. The values on the curve indicate the average number of young raised by the ten rats on each diet.

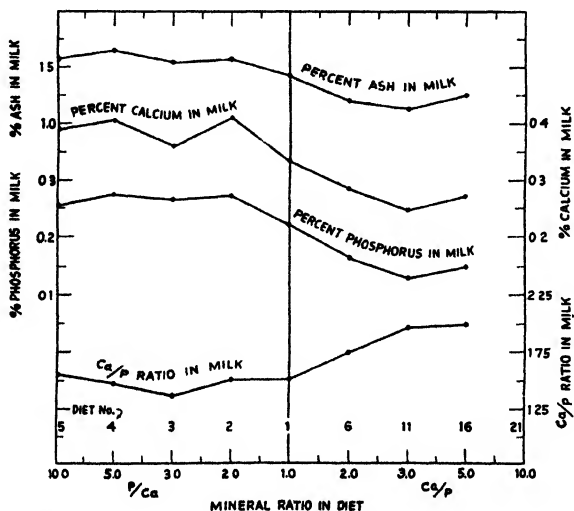


Fig. 3 The effect of changes in calcium and phosphorus levels of the maternal diet on the inorganic constituents of the milk.

a decrease in the absolute amounts of both calcium and phosphorus.

*Yeast.* To determine the effect of yeast, ten rats at parturition were placed on the basal diet (no. 1) save that the protein level was adjusted to 10% casein. Levels of 8, 4, 2, 1 and 0% yeast were used. The controls received 4% of a B-complex concentrate. Half the mothers were kept on wire screens during lactation, and the other half on shavings, but as no essential difference was observed in the behavior of these subgroups, the results have been averaged for presentation (fig. 4 and table 4).

TABLE 4

*Effect of decreasing yeast on the composition of milk.<sup>1</sup>*

DIET NO.	YEAST	FAT	PROTEIN	ASH
		%	%	%
60	4% Conc.	16.40 (19)	9.71 (20)	1.40 (18)
61	8	15.16 (8)	8.83 (8)	1.24 (8)
62	4	15.46 (14)	8.91 (14)	1.10 (13)
63	2	15.49 (7)	9.10 (8)	1.26 (7)
64	1	15.49 (10)	9.74 (10)	1.10 (8)
65	0	17.63 (8)	10.15 (8)	1.28 (6)

<sup>1</sup> The figures within parentheses indicate the number of determinations.

The limiting factor in all diets was the 10% casein level, and this accounts for the small size of young on 4% yeast concentrate, and 8% yeast. Differences from the findings on these two control diets are attributed to the level of yeast, although there was progressive reduction in the intake of yeast protein.

It is evident from figure 4 that decreasing amounts of yeast (the sole source of the B-factors in the diet) constitute a progressive drain on the maternal organism, as indicated by her loss in weight during lactation, a reduced volume of milk, and the size of young.

The fact that young could be raised on no yeast intake, during a period when the B-requirement is increased, is a point of considerable interest. It is generally believed that

vitamin B is not stored in the tissues so that the partial success in lactation on a B-deficient diet may be an expression of the time necessary for a B-deficiency to develop, or is related to the intestinal bacterial synthesis of B as recently reported by Najjar and Holt ('43). It should also be noted that the maximum effect of lack of yeast was in the loss in weight of the mothers, and in reduced milk volume rather than in any large weight difference in the offspring. The weight of young for 4, 2 and 1% yeast was the same, but this was accomplished at the expense of the weight of the mother.

Table 4 gives the percentages of fat, protein and ash in the milk. No significant change in any component was noted.

#### DISCUSSION

Scientific feeding for milk production is based on such a volume of work that it is not surprising that observations with rats do not disclose hitherto unrecognized facts. This applies particularly to the study of protein level, but it is of interest to know that the dietary factors affecting milk secretion in omnivora are similar to those which operate in herbivora. Thus, increase in protein intake does not materially change the per cent of milk protein, but does definitely increase milk volume. This is apparently true in cows, rats and humans. In our experiments increase in milk volume resulted in larger young but this could not be demonstrated in Adair's ('25) human observations (probably because of complementary feedings).

In spite of wide variations in mineral intake, the Ca/P ratio of rats' milk is maintained fairly constant between 1.4 and 1.7 although the total range was from 1.4 to 2.0. The chief effect of high calcium low phosphorus diets is not only to increase the Ca/P ratio of the milk but more significantly to decrease the total amount of calcium secreted. Thus, on diets numbered 1, 2, 3, 4, 5 and 6, a total of 5-10 mg. of calcium was secreted (under the technique described), while on diets 11 and 16, only 2.6 and 1.3 mg., respectively, was secreted. Thus, when



rachitogenic diets are fed, the young suffer most acutely from a calcium deficiency and therefore do not grow.

Corresponding to the low absolute amount of calcium secreted, the per cent of calcium in the milk on these high calcium rations was low (fig. 3). The number of determinations on each diet was not large (5-8) and individual variation in rats resulted in a large standard deviation. The differences between adjacent points (as between diets 1 and 6, and 6 and 11) have no statistical significance (significance ratios were 1.6 and 2.0, respectively) but the significance ratio for diets 1 and 11 was 3.1. The trend is sufficiently distinct to indicate the detrimental effect of high calcium diets on the calcium content of the milk. The food intake of lactating rats is not easily measurable but qualitative observations indicated that with the exception of diet no. 21 (table 3) the rats on the mineralized rations and on those containing different levels of yeast, consumed approximately the usual amount of food.

#### SUMMARY AND CONCLUSIONS

Milk from individual stock rats has been analyzed for protein, fat, ash, calcium, phosphorus and magnesium.

The effect of changes in the diet of lactating rats has been studied by observing the change in composition of the milk, the number and size of young raised, and the effect of the diet on the mother rat. It is concluded that:

1. Increase in the protein intake results in increased milk yield, but no significant change in the per cent protein in the milk.
2. High calcium diets result in decrease in both milk volume and the percentage of calcium and phosphorus in the milk, with consequent decrease in the total amount of calcium and phosphorus secreted. High phosphorus diets had no demonstrable effect on these milk constituents.
3. Decrease in the intake of yeast decreases the volume of milk, the size of nursing young, and causes severe weight loss in the lactating mothers, without changing significantly the composition of the milk.

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# THE NUTRITIVE VALUE OF THE PROTEIN IN TOBACCO-SEED OILMEAL<sup>1</sup>

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## THREE FIGURES

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Mitchell, Hamilton and Beadles ('45) have pointed out that the various seed meals, as potential sources of protein for nutrition, are too often neglected in the quest for higher yields of oil. Vickery et al. ('32) investigating the nutritive properties of seed from Connecticut shade-grown tobacco found the whole ground seed to support good growth in albino rats and mice. Their chemical separation of various nitrogenous fractions proves the presence of certain amino acids. Since tobacco-seed oilmeal contains 36% crude protein on the oil-free basis, investigation of the nutritive value of this potential source of dietary protein appeared desirable.

A cold-pressed meal, containing about 30% crude protein and 21% oil, was used in this investigation to avoid any possible alteration of the proteins during evaporation of the oil solvent.

## EXPERIMENTAL DATA

### *Growth promotion studies*

For the growth studies weanling rats weighing 40-50 gm were transferred from the stock colony to group cages and fed ad libitum the respective experimental diets. With two

<sup>1</sup> The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

exceptions each group of rats fed a given ration consisted of equal numbers of males and females.

The tobacco-seed oilmeal fed in both phases of the investigation was prepared by subjecting fine-ground seed to a pressure of 2500 lbs./sq. in. in a Carver press until no more oil was obtained. This fresh meal was incorporated immediately into rations or stored in the refrigerator until needed. Although the composition of various batches of pressed seed differed slightly, the following data, obtained upon the meal used for

TABLE 1

*Composition of rations used in determining the nutritive value of tobacco-seed as a source of protein.*

CONSTITUENT	RATION NUMBER									
	128	138	140	142	144	148	150	152	154	156
Tobacco-seed oilmeal <sup>1</sup>	60.0	33.3	33.3	33.3	33.3	33.3	33.3	33.3	33.3	50.0
Cornmeal		63.3			21.1					
Rolled oats			35.3		11.8					
Skimmilk powder				14.0	4.7					
Casein						5.0				
Lactalbumin							5.0			
Gelatin								5.0		
Wheat gluten									5.0	
Glucose	38.0		28.4	49.7	26.1	58.7	58.7	58.7	58.7	47.0
Salts <sup>2</sup>		2.4	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Sodium chloride	1.0									
Fortified corn oil <sup>2</sup>	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

<sup>1</sup> Prepared by pressing ground seed in a Carver laboratory press.

<sup>2</sup> For composition, see Skinner and McHargue ('45).

determination of the biological value of protein are typical: crude protein ( $N \times 5.34$ ),<sup>2</sup> 25.8%; ether extract, 21.5%; crude fiber, 24.5%; moisture, 5.0%. Composition of rations is given in table 1. These were prepared fresh at least weekly.

Because sex played no significant role in rate of growth of rats fed the various rations, growth curves in figures 1-3 show the average weight of both sexes.

<sup>2</sup> Vickery et al. ('32) advocate the use of 5.34 instead of the usual 6.25 due to the high nitrogen content of the principal globulin of tobacco seed.

Three groups of rats were fed tobacco-seed oilmeal as the sole source of protein. Two of these groups received different lots of ration 128, one of which contained tobacco-seed oilmeal which had been autoclaved for an hour at a pressure of 15 lbs./sq. in. Both rations contained 18% protein. The third group was fed ration 156 containing 15% unheated protein. In figure 1 it will be seen that rats grew about  $\frac{1}{3}$  faster at the 18% level of intake than at the 15% level. The average increases in weight during the first 6 weeks were 32 and 23 gm, respectively. The poor growth of the remaining group, 12 gm in 6 weeks, suggests that the proteins of tobacco seed were damaged by the heat treatment. The curve shows that no improvement resulted from addition of 0.3% cystine to the ration.

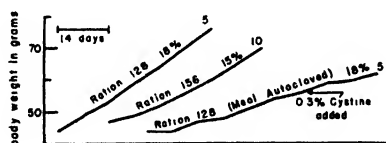


Fig. 1 Growth rate of animals with tobacco-seed oilmeal as the sole source of protein. First two curves show relationship of growth to level of protein intake. Third curve demonstrates detrimental effect of autoclaving the meal. Numerals above the curves indicate the number of animals fed each ration.

Since preliminary experiments demonstrated that proteins of tobacco-seed oilmeal alone support only slow growth, the next experiments were designed to ascertain the nature of the deficiency. Accordingly, four rations (148, 150, 152, and 154) were devised to demonstrate the supplementary value of casein, lactalbumin, gelatin and wheat gluten, respectively. They were incorporated at a level of 5% into rations containing sufficient tobacco-seed oilmeal to furnish 10% crude protein. It will be observed in figure 2 that fair growth was obtained on rations containing casein and lactalbumin supplements (rations 148 and 150, respectively). The response to supplementation with gelatin was less pronounced and was uninfluenced by addition of 0.5% cystine to the ration. Wheat gluten was even less satisfactory than gelatin as a supple-

ment for tobacco-seed protein. Of interest is the fact that during a period of 6 weeks the average gain of rats in the gluten group was 22 gm as compared with the gain, previously noted of 23 gm by rats consuming ration 156 in which 15% crude protein was furnished by tobacco-seed oilmeal alone. Since gluten is known to be deficient in lysine, it appeared probable that the proteins of tobacco-seed oilmeal were also deficient in this amino acid. That this is true was shown by the growth of six rats which for 4 weeks were fed ration 156 to which 0.5% of lysine monohydrochloride was added. During this period the average gain was 62 gm, exactly the increase in weight made by the rats fed the casein supplement (ration 148).

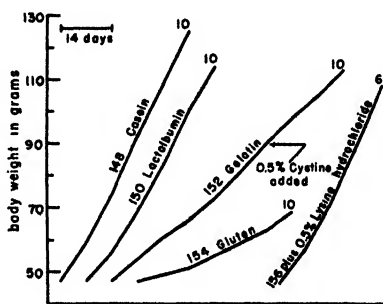


Fig. 2 Growth on tobacco-seed oilmeal with supplements of other proteins or lysine. First four curves represent animals fed tobacco-seed oilmeal at 10% protein level with 5% supplements of the proteins indicated. The last curve shows growth on tobacco-seed oilmeal at 15% protein level with addition of 0.5% lysine hydrochloride. Numerals above the curves indicate the number of animals fed each ration.

Use of a product such as tobacco-seed oilmeal as a major source of protein in rations can become practicable only if amino-acid deficiencies thereof are corrected by those materials which find frequent use in animal feeding. With this in mind, four additional rations (138, 140, 142, and 144) were fed to weanling rats for a period of 6 weeks. Each ration contained sufficient tobacco-seed oilmeal (33.3%) to furnish 10% crude protein. In three of them an additional 5% of protein was contributed by dried skimmilk, rolled oats, and

cornmeal, respectively. For the fourth ration (ration 144) these ingredients were added in such proportions that each contributed  $\frac{1}{3}$  of the supplementary protein. Since casein and lactalbumin when fed singly had proven to be effective supplements for tobacco-seed protein, it was expected that dried skimmilk would produce satisfactory response in growth. Upon examination of the growth curve for rats fed this supplement (fig. 3) it will be seen that the slope is almost the same as when casein was added at a level of 5% (fig. 2). Failure of cornmeal greatly to enhance growth on tobacco-seed protein

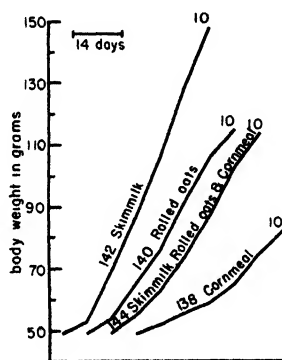


Fig. 3 Growth on tobacco-seed oilmeal with supplements of other protein-containing foods. All curves represent animals fed tobacco-seed oilmeal at 10% protein level with 5% protein supplements supplied by the foods indicated. In ration 144 each of the foods furnished  $\frac{1}{3}$  of the supplementary protein. Numerals above curves indicate the number of animals fed each ration.

was doubtless due to its low content of lysine. Rolled oats and the mixture of the three supplements were of approximately equal value in correcting the deficiencies of tobacco-seed protein.

#### *Determination of digestibility and biological value*

In order to ascertain the coefficients of digestibility and the relative biological values of the proteins of tobacco-seed oilmeal and milk, five pairs of young male rats were carried through the entire series of feeding periods, which lasted for



68 days. Each pair consisted of brothers of approximately the same initial weight. They were pair-fed (Mitchell and Beadles, '30) according to the reversal procedure of Mitchell et al. ('45) throughout the investigation.

The rats were housed in glass cages for collection of urine and feces. The cage proper consisted of a 1-gallon glass jug from which the bottom was removed by cutting off approximately 1 inch above the base. A tripod 5 inches in diameter and 9 inches high supported the inverted jug securely at a satisfactory height above the table.

The false bottom, cut from  $\frac{1}{2}$ -inch mesh wire, was suspended by wires from the rim of the inverted jug; the depth to which it was lowered into the jug depended upon the size of the rat. A circular piece of wire gauze 3 inches in diameter was laid above the neck of the jug to retain the feces. In most instances the feces were deflected to the gauze thereby preventing to a great extent subsequent contamination with urine. A loop of fine wire attached rigidly to the wire gauze served as a handle by which it could be conveniently lifted from the cage so as to facilitate collection of the fecal pellets. The neck of the bottle was provided with a tightly fitting rubber stopper carrying a glass tube of such length that it extended about 2 inches into the 200-ml Erlenmeyer flask which served as a receptacle for the urine. A small quantity of 0.1 N sulfuric acid in the latter prevented growth of bacteria and loss of ammonia. The feces were removed daily, at which time residual urine was removed from the walls by washing with a stream of hot 0.1 N sulfuric acid until the total volume in the receiving flask was approximately 150 ml. During a given period, urinary samples for each animal were filtered through glass wool to remove particles of feed, combined in a 2½-liter bottle and preserved with thymol. Daily fecal samples likewise were combined and preserved under 95% alcohol containing a few drops of sulfuric acid. Collection periods, which were of 7 days' duration, were preceded by transition periods either 3 or 5 (usually 5) days in length. All nitrogen determinations on feces, urine and rations were made according

to the Gunning method in the Official and Tentative Methods of Analysis ('40) with the use of selenium as a catalyst as described by Lauro ('31).

Composition of the rations used in the determination of biological values is given in table 2. In order to eliminate variables among these rations (161A, 161B, 161C and 161D) sufficient tobacco-seed oil and Cellu Flour<sup>2</sup> were incorporated into 161B and 161C to raise the fat and fiber content to that of the two rations containing tobacco-seed oilmeal. All ra-

TABLE 2

*Composition of rations used in the determination of biological values.*

CONSTITUENT	RATION NUMBER			
	161A	161B	161C	161D
Tobacco-seed oilmeal <sup>1</sup>	33.1			
Skim milk powder		29.8		
Dried egg <sup>3</sup>			5.4	
Glucose	63.0	38.8	61.1	
Salts <sup>2</sup>	2.6	1.6	3.7	
B vitamins <sup>2</sup>	0.3	0.3	0.3	
Fortified corn oil <sup>2</sup>	1.0	1.0	1.0	
Tobacco-seed oil <sup>1</sup>		7.1	7.1	
Cellu Flour		21.4	21.4	
Ration 161A				99.5
l(+ )lysine monohydrochloride				0.5

<sup>1</sup> Prepared by pressing ground seed in a Carver laboratory press.

<sup>2</sup> For composition, see Skinner and McHargue ('45).

<sup>3</sup> Dried below 60°C. and extracted with ether.

tions contained approximately 10% protein except the standardizing egg ration (Mitchell and Carman, '26) in which the concentration of protein was only 4%. Each ration was prepared in quantity sufficient for the entire metabolism period and stored in the refrigerator.

Since it has been reported by Mitchell ('24a) that the metabolic nitrogen and endogenous urinary nitrogen of a given rat change with time, each animal was placed on the

<sup>2</sup> A product obtained from the Chicago Dietetic Supply House, containing 37.3% crude fiber.

standardizing egg ration once near the beginning and once near the end of the experiment. In this way two values were obtained and plotted which permitted a specific value to be interpolated or extrapolated for each animal during the test period under observation.

From the nitrogen metabolism data taken the coefficients of true digestibility and biological values were computed in the usual way. The values thus obtained are reported for

TABLE 3

*A comparison of the digestibility and biological value of the protein of tobacco-seed oilmeal with the protein of skim milk powder.*

RAT NO.	TOBACCO-SEED OILMEAL		TOBACCO-SEED OILMEAL PLUS LYSINE		SKIMMILK POWDER	
	True digestibility	Biological value	True digestibility	Biological value	True digestibility	Biological value
1	72 <sup>1</sup>	60 <sup>1</sup>			99	83
2	78 <sup>1</sup>	49 <sup>1</sup>			98	87
3	78 <sup>1</sup>	58 <sup>1</sup>			100	80
4	78 <sup>1</sup>	53 <sup>1</sup>			96	89
5	82 <sup>1</sup>	54 <sup>1</sup>			96	76
6	78	47	77	74	95 <sup>1</sup>	65 <sup>1</sup>
7	77	47	75	72	92 <sup>1</sup>	78 <sup>1</sup>
8	79	52	80	70	97 <sup>1</sup>	80 <sup>1</sup>
9	79	45	79	79	96 <sup>1</sup>	72 <sup>1</sup>
10	79	49	82	74	97 <sup>1</sup>	75 <sup>1</sup>
Avg.	78.0	51.4	78.6	73.8	96.6	78.5
Avg. 1-5	77.6	54.8			97.8	83.0
Avg. 6-10	78.4	48.0	78.6	73.8	95.4	74.0

<sup>1</sup> Average values of two or more test periods.

each animal in table 3. Averages of the individual values are recorded whenever an animal was fed a given ration during more than one period.

#### DISCUSSION OF RESULTS

Although a ration containing tobacco-seed oilmeal as the sole source of protein would be incomplete, the results indicate that this material may serve satisfactorily as a source of

dietary protein when fed in combination with other materials of moderately high lysine content. As Mitchell ('43) has pointed out, the value of a given protein as a supplement is probably more important than its biological value alone since few diets contain only one source of protein.

In the digestibility determinations the relatively high fiber content (8%) apparently had no effect upon the true digestibility since the values for skimmilk powder are in agreement with those reported by Mitchell ('24 b). This fact was also observed by Adolph and Wu ('34) using much higher concentrations of roughage. Results in table 3 show that rats 1-5 were, as a group, more efficient in utilization of protein consumed than their pair mates. The average biological value of tobacco-seed oilmeal protein when calculated from data obtained upon these rats was 54.8 as compared with 48.0 for rats 6-10. With skimmilk protein the biological values observed in the two groups as named were 83.0 and 74.0. Considering rats 6-10 since they only were fed the ration containing added lysine, it will be seen that this amino acid did not change the true digestibility but raised the biological value of the oilmeal protein from 48.0 to 73.8, approximately that found for skimmilk protein when fed to the same rats. These numerical values explain why the slopes of the growth curve for lysine supplemented ration (fig. 2) and that for the ration supplemented with skimmilk (fig. 3) are equal.

Mitchell and Carman ('24) have called attention to the superiority of "net protein" value to either digestibility or biological value alone as a basis for rating sources of dietary protein. Total protein content as well as the two other factors is involved in the calculation of this value. Pressed tobacco-seed oilmeal above gives a net protein value of 10.8% and with lysine 16.7%. Assuming no change in digestibility or biological value tobacco-seed oilmeal (extracted at less than 70°C.) gives a net protein value of 14.3%. Comparison with the net protein values of 21.3% for raw soy flour (Mitchell et al., '45), 10.6, 5.0, 3.5, and 6.0% for rolled oats, white flour, whole corn and navy beans, respectively, (Mitchell, '27) proves

the above forms of tobacco-seed oilmeal to rank high as a source of dietary protein.

The results of a previous investigation (Rapp et al., '46) show that although use of the cold-pressed oilmeal with its residual oil content was suitable for these experiments, the commercial storage problem created by the susceptibility of the oil to oxidation would very probably necessitate utilization of oil-free meal as a source of dietary protein.

#### SUMMARY

1. When tobacco-seed oilmeal was fed to rats at levels of 15 to 18% protein, the rats made only slow growth. The oilmeal was proved to be deficient in lysine.

2. When a ration containing tobacco-seed oilmeal as the source of protein was supplemented with casein, lactalbumin, skimmilk powder, or lysine, good growth was obtained. Rolled oats, cornmeal and gelatin were less satisfactory supplements to the oilmeal protein. Addition of wheat gluten was no more effective than increasing the proportion of tobacco-seed oilmeal so as to equalize the protein content of the two rations.

3. The biological values of the protein of tobacco-seed oilmeal and skimmilk powder when fed at a level of 10% were 51.4 and 78.5, respectively. Values for true digestibility of the two in the order named were 78.0 and 96.6. The biological value of the oilmeal protein when supplemented with lysine was approximately that of milk proteins.

4. Because of its relatively high net protein value tobacco-seed oilmeal deserves consideration as a source of dietary protein.

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# THE NUTRITIVE VALUE OF TOBACCO-SEED OIL<sup>1</sup>

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## TWO FIGURES

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Under present methods of producing tobacco in Kentucky, seed are used only for starting the following year's crop. However, with the increased practice of priming tobacco it is likely that a considerable quantity of seed could be produced without seriously affecting the quality of the leaf, provided a sufficient demand for the byproducts from the seed were created.

Mendel and Vickery ('32), investigating whole ground tobacco seed, obtained good growth with albino rats and mice even when the diet was 99% ground tobacco seed. Previous investigations at this Station by Scherffius and Woosley ('09) and McHargue et al. ('42) show that Kentucky tobacco seed contains about 40% of oil which has drying properties.

Although association of drying oils and nutrition is uncommon, Kaufmann ('41) reported that oil from the seed of certain European tobaccos can be used in foods. The purpose of this investigation was to ascertain the nutritive value of the oil from the seed of *Nicotiana tabacum*, which is commonly grown in Kentucky.

## EXPERIMENTAL DATA

Before starting the feeding experiments, tobacco seed was analyzed for nicotine and nornicotine. Neither of these sub-

<sup>1</sup> The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.



stances was found. This is in agreement with the results reported by Vickery and Pucher ('30) investigating the mature seed of Connecticut shade-grown tobacco. Il'in ('29) found that immature seed contained small amounts of the alkaloids and Mendel and Vickery ('32) reported the detection of nicotine in the sprouts and cotyledons after 9 to 11 days germination. However the latter investigators go on to show that this has little or no effect upon the nutritive properties of the seed.

The composition of each experimental ration used in this investigation is shown in table 1.

TABLE 1  
*Composition of rations (per cent).*

CONSTITUENTS	RATION A	RATION B	RATION C	RATION D	RATION E
Fat or oil <sup>1</sup>	0	5	15	30	30
Casein <sup>2</sup>	18	19.1	21.4	24.7	24.7
Glucose	76.7	70.3	57.3	38.2	38.2
Salt mixture <sup>3</sup>	5.0	5.3	6.0	6.8	6.8
Vitamin supplements <sup>4</sup>	0.3	0.3	0.3	0.3	0.3

<sup>1</sup> Butterfat was obtained from melted creamery butter. The cotton-seed oil was a refined product sold for human consumption. Total tobacco-seed oil used in rations B-D was obtained by extraction with a low-boiling petroleum fraction, evaporation of the solvent and partial bleaching with fuller's earth. Ration E related only to pressed tobacco-seed oil which was untreated.

<sup>2</sup> Ratio of protein to energy intake remained constant throughout.

<sup>3</sup> Approximately that of Phillips and Hart ('35). Ratio of salts to energy intake remained constant throughout.

<sup>4</sup> Skinner and McHargue ('45) except that fat-soluble vitamins were dissolved in ether.

### *Digestibility determinations*

Digestibility coefficients of tobacco-seed oil, cotton-seed oil and butterfat were determined with ad libitum feeding at three levels of fat intake on three groups of four young adult male rats, housed in individual wire cages. Each digestion period lasted 9 days and consisted of a 2-day transition period followed by 7 days during which food consumption was recorded and feces were collected daily, placed in bottles and kept in a refrigerator. During the initial digestion period each ani-

mal was fed fat-free ration A. In subsequent periods rations B, C and D (containing 5, 15 and 30% fat, respectively) were fed in the order named, one group of animals receiving tobacco-seed oil at the three levels, another cotton-seed oil and the third butterfat. All rations were kept in the refrigerator. At the end of a given 7-day collection period the pooled feces of each animal were dried at 100°C. and extracted with ether to determine the amount of fat excreted. The fat excreted on ration A was considered metabolic fat and the relationship between metabolic fat and fat-free food intake was assumed

TABLE 2  
*Coefficients of digestibility (per cent).*

OIL OR FAT IN RATION	AT 5% LEVEL		AT 15% LEVEL		AT 30% LEVEL	
	Average	Range	Average	Range	Average	Range
Tobacco-seed oil	97.6	97.5-98.8	97.8	97.2-98.2	98.2	98.0-98.4
Cotton-seed oil	99.4	99.0-99.5	99.0	98.9-99.2	99.0	98.7-99.2
Butterfat	98.4	97.7-99.2	97.7	95.5-98.8	98.4	97.2-98.9

TABLE 3  
*Iodine number (Hanus).*

OIL OR FAT IN RATION	INGESTED FAT OR OIL	FAT EXCRETED BY GROUP WHEN FED			
		No fat	5% fat	15% fat	30% fat
Tobacco-seed oil	148.3	51.6	47.4	42.8	44.5
Cotton-seed oil	103.1	54.4	60.7	56.1	56.9
Butterfat	30.2	44.4	36.6	38.8	38.5

to remain constant in subsequent periods as was done by Langworthy and Holmes ('15). The average coefficients of digestibility, as well as the ranges, for each fat intake level are given in table 2. Though cotton-seed oil showed a slight margin over the other two fats, there was very little difference between any of these fats when fed at the same level or the same fat when fed at different levels up to 30%.

The iodine number (Hanus method) was determined on each of the fats used in the rations and likewise on the composite samples of fecal fat excreted during the respective metabolism periods. Results are shown in table 3. Assuming the

composition of Kentucky tobacco-seed oil to be similar to that of the oil from European tobaccos analyzed by Lesyuis ('40) and Kaufmann ('41) and using the predicted digestibilities of Holt et al. ('35) the results in tables 2 and 3 were as anticipated.

### *Growth promotion studies*

For the study of growth promotion, rations C and D of each fat were fed ad libitum to three matched groups of weanling rats (four male and four female) for a period of 4 weeks. Bulk rations were stored in closed jars at room temperature

TABLE 4  
*Growth data (ad libitum feeding for 4 weeks).*

OIL OR FAT IN RATION STORED AT ROOM TEMPERATURE (DURING AUG., 1944)	TOTAL WEIGHTS OF GROUPS (GM)					
	Group of 4 males			Group of 4 females		
	Initial	Final	Gain	Initial	Final	Gain
At 15% level						
Tobacco-seed oil	176	179	3	176	215	39
Cotton-seed oil	181	412	231	176	365	189
Butterfat	176	616	440	177	506	329
At 30% level						
Tobacco-seed oil	178	<sup>1</sup>		173	<sup>2</sup>	
Cotton-seed oil	177	706	529	173	499	326
Butterfat	178	687	509	172	533	361

<sup>1</sup> All animals in group lost weight and died. Average survival was 12 days.

<sup>2</sup> All animals in group lost weight and died. Average survival was 11 days.

(during August 1944). Results are given in table 4 as total-initial and -final weights for each group as well as total weight gained by the group. The animals receiving butterfat made the largest gains while those on tobacco-seed oil rations made the least gains. At the 15% level average weekly gains on tobacco-seed oil were almost negligible and at the 30% level all animals died before completion of the experiment as indicated by their average time of survival shown in table 4.

In view of the poor gains made by the animals on tobacco-seed oil rations, a duplication of this part of the experiment

was made using eight additional animals at the 15% level and six animals at the 30% level. The previous results were confirmed and every animal which survived the entire experiment showed erratic weight changes.

It was observed in the foregoing experiments that after storage for a short time at room temperature the tobacco-seed oil rations became spongy and had a pronounced odor even though the oil used in making the rations had been stored in closed bottles at room temperature for several months with no evidence of becoming rancid. This indicated that the rate of oxidation of the oil was increased quite rapidly by the higher temperatures and greatly enlarged surface area. Since the latter could not be reduced materially, control of temperature provided the only convenient means of retarding oxidation.

Preliminary tests showed that about half of the oil contained in seed could be expressed in a relatively short time by a Carver laboratory press at maximum pressure. The oil obtained in this way possessed the same physical and chemical constants as the bleached extracted oil. Substitution of this oil in subsequent experiments made possible a supply of freshly prepared oil and also eliminated the heat treatment necessary to remove the solvent from the extracted oil.

To determine the extent to which the nutritive value of the ration was affected by development of rancidity, two groups of six young rats each were fed ration E. One group was fed from bulk ration stored at room temperature while the ration for the other group was refrigerated. The rats were fed daily from the respective stocks and any unconsumed ration from the day before was discarded. Individual growth curves for these animals over a period of 5 weeks are shown in figure 1. Attention is called to the second break in the curves at the right demonstrating a pronounced response to freshly mixed ration. Also it might be mentioned that animals 1 and 4 in the curves at the right were started together on fresh ration, animals 2 and 5 were started 3 days later being fed from the same bulk ration and 3 and 6 were started 6 days still later, on the

ration corresponding to that of the second week of the first pair. The growth curves of the pairs, showing the extremes in growth as related to age of the ration, are similar.

In their review Burr and Barnes ('43) point out that rancid fats catalyze the destruction of certain fat-soluble vitamins. In view of the previous experiments it seemed necessary to determine whether the poor growth of animals on tobacco-seed oil ration was due primarily to resultant vitamin deficiencies.

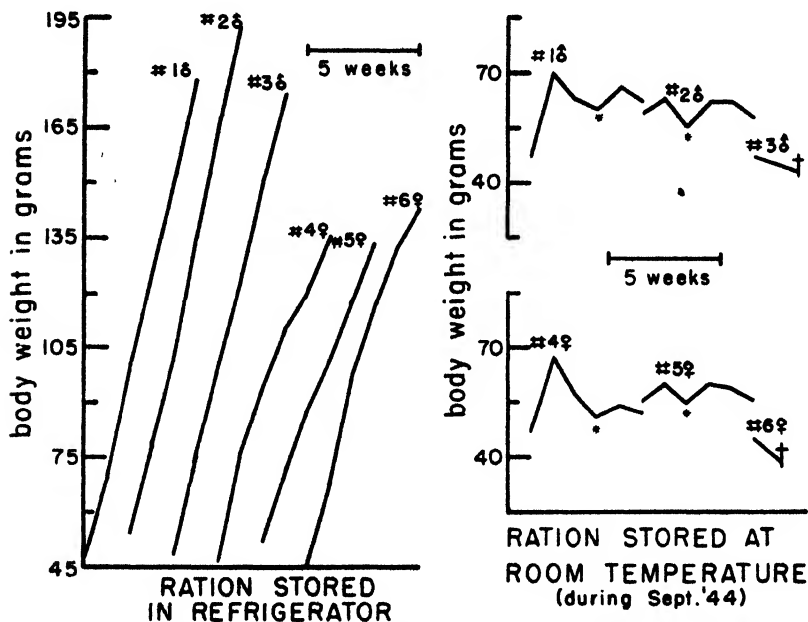


Fig. 1 Individual growth curves of animals on 30% pressed tobacco-seed oil ration. \* Freshly mixed ration fed at this point. † no. 3 ♂ died in 14 days, no. 6 ♀ died in 9 days.

Consequently, seven young animals (ca. 3 weeks old) were put on total oil ration D stored at room temperature. In addition, two of the animals received a daily supplement of fat-soluble vitamins, two other animals received B vitamins and three animals were fed both fat-soluble and B vitamins. These daily supplements were given individually in separate containers and in amounts equivalent to those furnished by 10 gm

of freshly prepared ration. All animals consistently lost weight and survived for periods varying from 4 to 28 days.

Comparison of the average growth rates of animals on butterfat ration D and refrigerated tobacco-seed oil ration E, 31.8 and 26.2 gm/week, respectively, for males and 22.6 and 17.9 gm/week, respectively, for females, indicates better growth on butterfat with ad libitum feeding. However, it was observed that the animals of the former group consumed more ration than those on the tobacco-seed oil ration. Consequently, twelve rats were paired as closely as possible with regard to initial weight, sex and litters and fed controlled

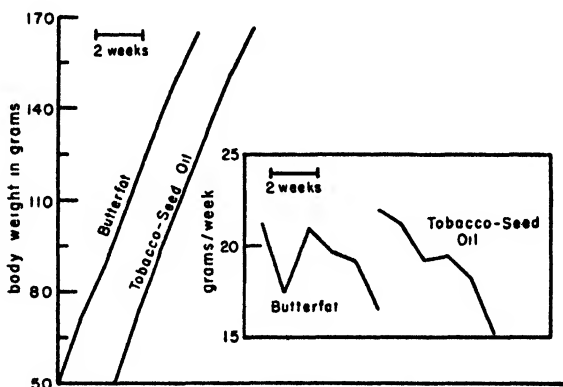


Fig. 2 Average growth of six animals on 30% ration stored in refrigerator (paired-feeding). Insert shows average weekly gains.

amounts of butterfat ration D and ration E (refrigerated) for a period of 6 weeks. During the first 2 weeks of the experiment the animals on tobacco-seed oil established a lead in total weight gained, part of which was maintained for the entire 6 weeks. Over the whole period differences in weight gains of the individual pairs were insignificant except in one instance. The food intake of this pair was definitely limited by the animal on the butterfat ration. Average cumulative growth and weekly gain curves for the six pairs are shown in figure 2 and insert, respectively.

*Effect on functional activity*

In view of the possible destruction of certain of the fat-soluble vitamins in the tobacco-seed oil rations, the animals surviving the experiment on refrigerated and non-refrigerated tobacco-seed oil rations (fig. 1) were put on stock ration in mated pairs designed to determine if reproduction had been permanently impaired in either the males or females by either of the experimental diets. Those animals which had received the unrefrigerated ration and were most likely to be sterile were paired with animals of known fertility from the stock colony. At the time of taking the animals off the experimental diets they had not reached maturity with respect to growth, those on non-refrigerated ration averaging only 55 gm. Since sexual maturity is related to growth maturity a delay in production of offspring was expected. This proved to be the case with all the animals and especially so with the extremely small ones. However, eventually all animals proved to be fertile.

## DISCUSSION OF RESULTS

Results of the experiments show that, for all practical purposes, the coefficients of digestibility of the three fats investigated are high and equal and, under certain feeding conditions, the fats support good growth in young animals. The observation that growth of pair-fed rats was the same on rations containing butterfat and tobacco-seed oil, respectively, is in keeping with the comparative nutritive values of butterfat and other vegetable oils reported by Deuel et al. ('43). It is doubtful if flavor contributed to the superiority of butterfat under ad libitum feeding since unrefined tobacco-seed oil possesses no particularly objectionable flavor. Moreover, Boutwell et al. ('44) found that removal of flavoring agents from butterfat or addition of diacetyl to corn oil had little effect upon the comparative nutritive values of these two fats.

Temperature and exposed surface area have a pronounced effect on the rate of oxidation of tobacco-seed oil and in this

respect it follows the pattern of other drying oils. However, if certain precautions are taken when the oil is most subject to oxidation (i.e., after mixing with other foods so that a large area is exposed) there is little reason for it being unsuited for dietary purposes. Even these precautions are unnecessary as long as the oil is isolated and stored in closed containers. Although this property is less pronounced with the commonly accepted edible fats and oils it is well recognized that refrigeration is essential when these are incorporated in food mixtures.

The poor performance of the animals on unrefrigerated rations was not due primarily to destruction of vitamins present as the vitamin supplement experiment showed. Also no permanent ill effects were noted which might indicate severe vitamin deficiencies when proper precaution was taken.

#### SUMMARY

1. When tobacco-seed oil was fed to rats at levels of 5, 15 and 30% of the respective rations it gave an average coefficient of digestibility of 97.9 as compared with 99.1 and 98.2 for cotton-seed oil and butterfat, respectively.

2. With paired feeding of refrigerated rations containing 30% of tobacco-seed oil and butterfat, respectively, growth rates of rats did not differ significantly. When fed *ad libitum*, the difference in consumption of the two rations produced a greater rate of growth in rats fed butterfat.

3. Of six rats fed refrigerated ration and four animals fed non-refrigerated ration containing 30% tobacco-seed oil for 5 weeks, during the period when growth is usually maximum, all proved to be fertile upon reaching maturity.

4. Both temperature and surface area affected the rate of oxidation of the oil. When surface area was small, as in the case of the isolated oil, no particular care in storing was necessary; but when the oil was incorporated in a ration and kept for any length of time, refrigeration was necessary.



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# MENTAL RESPONSE TO ADDED THIAMINE

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## SIX FIGURES

(Received for publication May 31, 1945)

The present paper gives a short<sup>1</sup> account for the nutritionist of an experimental study of psychological response to liberal thiamine intake in children of school age. The study employed all the suitable children of the 120 living in the Presbyterian Orphans' Home at Lynchburg, Virginia. Without their knowledge, they were assigned to two groups matched very closely in pairs as to age, sex, stature, mental ability and educational achievement. All the children subsisted at the same table without segregation but the children of one group received daily a tablet containing 2 mg of thiamine while those of the other group received daily an indistinguishable placebo.<sup>2</sup> Measurements were made at regular intervals of the progress of the children in mental and physical skills by a systematic program of tests.

## GENERAL PROGRAM OF THE STUDY

The study covered three periods: (1) Six weeks' trial of thiamine supplement utilizing a wide range of test tasks, May 15–July 1, 1941 (Harrell, '43). (2) First year-long experiment with selected tasks, September 1942–September

<sup>1</sup> To be published in full under the title "Further Effects of Added Thiamin on Learning and other Processes," Bureau of Publications, Teachers College, Columbia University, New York.

<sup>2</sup> Tablets donated by Hoffmann-La Roche, Incorporated.

1943. (3) Second year-long experiment involving interchange of some children between groups, September 1943–September 1944. Child personnel in the orphanage gradually changed during the course of the experiments, thus somewhat limiting the number of matched pairs for which comparisons could be made during the longer periods. All the children participated in the program insofar as they were able and their scores were recorded to aid in maintaining the morale of the experiment. However, certain of the tests require ability to read. Full comparisons of performance are therefore largely limited to the age range 9 to 19 years. The actual number of pairs participating in all tests at each interval is indicated in tables 2 and 3. It varied from twenty to fifty-five pairs.

### *Orphanage regimen and dietary*

The orphanage is located on a 200-acre farm on which the children live, work and attend school. Food is prepared for all the children in a single kitchen and served in a single dining room at eighteen tables, each accomodating one supervisory adult and seven children. For economy the proportion of cereals in the dietary is high and that of meat is low. A gallon of excellent quality milk from a pure-bred Jersey herd which is tended by the older boys of the Home, is, however, served at each table at every meal. Butter and cheese from the farm dairy are liberally available. Menus are, however, repetitive offering little variety; those of October 18, 19, 20 and 21, 1942, are listed in table 1 as typical.

### *Thiamine in the diet*

Dietary surveys by nutritionists<sup>3</sup> of Columbia University were made during several days in June 1941 and again in October 1942. Weights of all foods eaten in the orphanage on the survey days were taken and the thiamine intake per child was calculated from tables (Taylor, '42) as 0.9 and 1.0 mg, respectively, per day during the two periods. No deduction

<sup>3</sup> Dr. Mary Robertson and Miss Eunice Peterson.

TABLE 1  
*Illustrative menus of the orphanage. (October 18, 19, 20 and 21, 1942.)*

DAY	BREAKFAST	DINNER	SUPPER
Sunday	Corn flakes; biscuits, baking powder, white flour (2/person); apple sauce; butter; milk	Ham, cold, boiled (1 slice per person); green beans, canned; potato salad; bread, whole wheat (2 slices per person); pineapple cake; milk	Peanut butter sandwich, white bread (1/person); cherry preserves sandwich, white bread (1/person); cookie (1/person); milk
Monday	Oatmeal; biscuits, baking powder, white flour (2/person); butter, milk	Vegetable soup; biscuits, baking powder, white flour (2/person); bread pudding; milk	Pork and beans, canned; cheese, 1 oz./person; biscuits, baking powder, white flour (2/person); milk
Tuesday	Oatmeal; molasses, canned; biscuits, baking powder, white flour (2/person); butter; milk	Dried beans, stewed; turnip greens; corn bread, white meal (1 square/person); milk	Spaghetti; salmon, cold, canned; biscuits, baking powder, white flour (2/person); apple sauce, milk
Wednesday	Corn flakes; biscuits, baking powder, white flour (2/person); butter; milk	Green beans, canned; mashed potatoes; tomatoes, stewed; corn bread, white meal (1 square/person); milk	Rice, white, boiled; carrot strips, raw; biscuits, baking powder, white flour (2/person); milk

was made for possible excessive cooking losses. During 1943-1944, in spite of precautions, enriched flour was encountered on two occasions and may have been used for a considerable period.

### *Parity of groups*

Immediately prior to each experimental period, data were taken for each individual child including height, weight, age, sex, educational achievement, length of residence in the institution and his scores in two intelligence tests, namely, the I.E.R. Intelligence Scale CAVD and either the Otis Tests of Mental Ability (first period) or the Kuhlmann-Anderson Intelligence Tests (second and third periods).

On the basis of these data, the children were assigned to two well-matched groups by Dr. Ella Woodyard and Dr. Grace MacLeod, psychologist and nutritionist, respectively, of Teachers College, Columbia University, neither of whom participated in the subsequent observations of the children. Choice of the group to serve as control and that for experiment was also made by these individuals and was kept secret from the children and from all adults who participated in measurements of the children. Possibility of subjective bias was thereby avoided.

Assignment of children was primarily such as to match closely the mental abilities in each group, child for child. It was also possible to adjust assignments to secure comparability in age, size, sex, educational status and ratio of weight to expected weight. Eighty per cent of the children in this orphanage have one or more siblings there, so it was often possible to secure representation in each group of identical parentage and, accordingly, similarity of early home life and economic background. The groups as finally constituted were so similar that no factor known in advance offered a basis for anticipating differences in achievement.

### *Supplementation procedures*

After secret assignment of the children and secret choice of the experimental group, Drs. Woodyard and MacLeod

placed in a series of envelopes, each bearing the name of a child, a suitable number of tablets of the appropriate sort whether a placebo or an indistinguishable tablet containing 2 mg of thiamine. These envelopes were dispatched to the orphanage where the matron of each dormitory cottage took custody of the appropriate ones and administered a tablet daily, usually at bed time, to each child from the envelope marked with his name. The supply of tablets was replenished as required in the same manner so that no participant in the study, either child or adult, could distinguish the experimental subjects from the control subjects, or could say, for example, whether Tom and Dick belonged to the same group or to different groups.

### *Measurements of performance*

In the 6 weeks' trial from May 15 to July 1, 1941, the tasks were numerous and varied and were performed at frequent intervals, the children working at them daily. During the two experiments of year-long duration, the number of tasks was reduced to those which previous experience had indicated were most satisfactory and informative. All tasks were, however, such as are recognized and used currently by psychologists. In all cases, tasks were selected to be well within the range of the children's abilities, to engage and hold their interest and to be capable of objective measurement. Selection of the tasks to be used was made with the advice of Dr. Arthur I. Gates, Dr. Helen M. Walker, Dr. Robert L. Thorndike and Dr. Ella Woodyard, all of the Psychology Department of Teachers College, Columbia University.

Tests of the selected activities were administered by persons of professional experience in their use. The intelligence tests were administered by Dr. Woodyard, Dr. Cora Friedline of Randolph-Macon Woman's College and Miss Loulie Burner of Asheville College. Dr. Lyman Abbott of the Wilmer Ophthalmological Institute of the Johns Hopkins Hospital and University, assisted by Miss Lelia Robertson, tested the children in visual efficiency with the Snellen chart. The tests

of binocular vision<sup>4</sup> were made by Misses Todd and Dameron of the Lynchburg public schools. The measures of height and weight and the incidence of colds among the children were recorded by the principal and the assistant principal of the orphanage school.

During the 6 weeks' trial the numerous and varied tasks were repeated nine times by each child. These tasks are indicated at the bottom of figures 1 and 2. The nature of most of the tasks will be evident but a few require description. "Problems" signifies Monroe's Standardized Reasoning Tests in Arithmetic. "Mixed Fundamentals (Thorndike)" involve addition and subtraction; those of Woody-McCall involve addition, subtraction, multiplication and division. "Sums of Ten" consist of encircling two adjacent numbers whose sum is ten on a page of printed numbers. "Completion" signifies completion of fifteen simple geometric designs. "Number Span" consists of reproducing in a designated space on the obverse side of the paper series of numbers printed on the face. "Code Learning" or "Code Substitution" refers to the writing of the proper associated digit beneath each of five geometric symbols recurring repeatedly but in random order on a page of printed symbols. "Right hand" and "left hand" refer to strength of grip as measured by a dynamometer. Time limits were progressively shortened for successive trials of the timed tasks except for Code Substitution which was invariably practiced 5 minutes each time throughout all experiments.

A modified program of tests was adopted for the longer term experiments, as required by considerations of expense, availability of expert personnel and the morale of the children during prolonged repetitive drill. The schedule of tests was that indicated at the bottom of figure 3.

Some explanations of these choices are required. The Kuhlmann-Anderson Intelligence tests were substituted for the Otis Intelligence tests used in the earlier period because

<sup>4</sup> Keystone Telebinocular was loaned by the public school system of Lynchburg, Virginia.

they were better adapted to the younger children who were included in the longer experiments. Due to the withdrawals of children from the Home as they reached the age of self-support, a process which was much hastened by war conditions including the draft for military service, the average age of

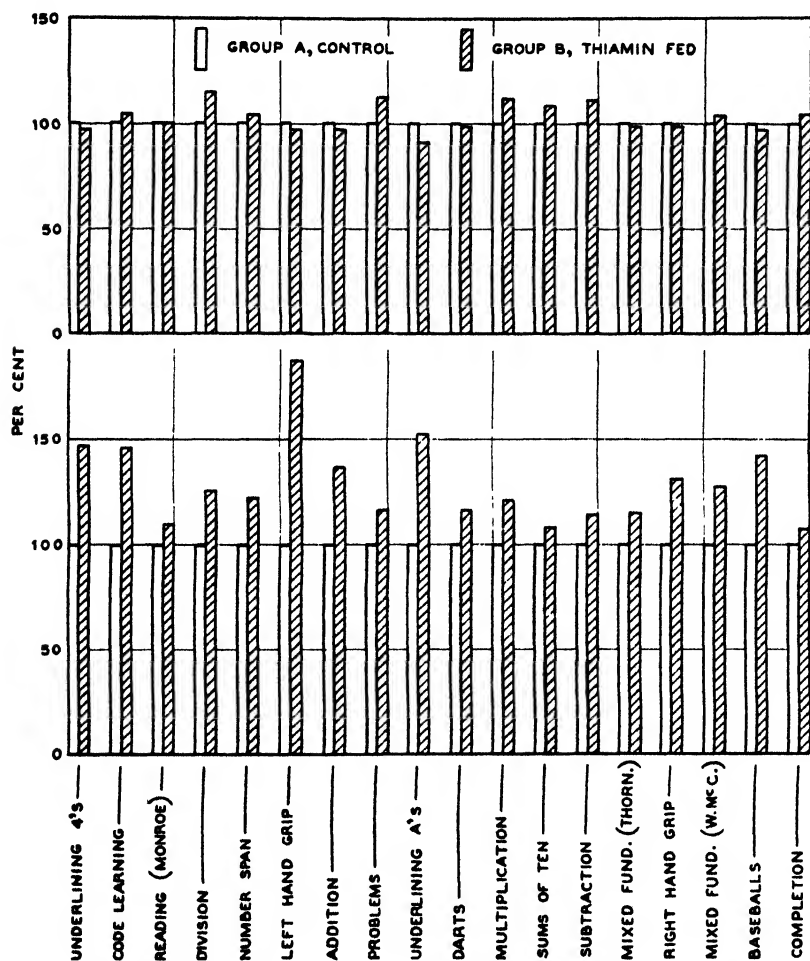


Fig. 1 (Upper graph) Mean standing of groups A and B at beginning of experiment (letting mean initial score of group A be 100%).

Fig. 2 (Lower graph) Relative mean gains made by groups A and B in 6 weeks of practice (letting gain of group A be 100%).



the available subjects was approximately 2 years younger during the later longer term experiments. Withdrawals progressively reduced the number of pair comparisons which could be made at successive intervals.

Visual perception tests were emphasized in the long experiments for the sake of including objective measurement of a sensory function. Expert assistance was available for visual tests but not for auditory.

Reaction time could not be measured during the earlier trial period for lack of suitable equipment. It was, however, included in the later program because of the high significance attached to it by professional psychologists.

Code Substitution was one of the original tests of which the children did not tire. Consequently it was carried on during the two later full year periods as well.

Incidence of colds and changes in height and weight would obviously be highly unreliable measures when taken over a 6 weeks' period but it was felt that they should be observed over the longer period of confirmatory experiments. Likewise changes in Educational Achievement could be measured only over the longer period, a consideration which also applies in some degree to the memory tests.

## RESULTS

### *Six weeks' trial*

Figures 1 and 2 (Harrell, '43) show, respectively, the comparative average performance of the two groups of thirty-seven children each at the beginning of the experiment and their relative gains at the end of 6 weeks of practice. In the eighteen test tasks which were repeatedly practiced by both groups at the same time and for the same length of time, it appeared significant that the vitamin fed group surpassed the control group in average gain in every task. The superiority of gain ranged from 7% to 87% and averaged 27% for the program of eighteen practiced tasks as a whole.

Statistically there were large individual variations of gain within each group. The significance of the mean difference

was computed for each task in the program after first finding the difference in gain separately for each of the thirty-seven pairs of children who performed all the tasks. The t-ratio which is the ratio of the mean to its standard error, (Student, '25) is given in table 2 for the differences in gain exhibited by the pairs of children in each of the eighteen activities. For seven of the tasks the significance is high; for eleven tasks the

TABLE 2

*Superiority of thiamine fed group B over control group A in gain during 6 weeks' trial period, May-July, 1941. (37 pairs of children.)*

ACTIVITY	T-RATIO	PROBABILITY THAT SUPERIOR GAIN WAS DUE TO CHANCE
1. Underlining 4's	3.33	0.002
2. Code learning	3.13	0.004
3. Reading (Monroe)	2.17	0.04
4. Division	2.11	0.04
5. Number span	2.09	0.04
6. Left-hand grip	2.07	0.04
7. Addition	2.07	0.04
8. Problems in arith.	2.00	0.05
9. Underlining A's	1.33	0.2
10. Darts	1.28	0.2
11. Multiplication	1.07	0.3
12. Sums of ten	1.03	0.3
13. Subtraction	0.67	0.5
14. Mixed fund. (Thorndike)	0.65	0.5
15. Right-hand grip	0.63	0.5
16. Mixed fund. (Woody-McCall)	0.61	0.5
17. Baseballs	0.60	0.5
18. Completion	0.54	0.6

t-ratios are small enough to permit the assumption that the measured superiorities may have been due to chance. However, it is almost inconceivable that by chance alone all eighteen superiorities should be in the same direction.

#### *First year-long experiment, 1942-1943*

Prior to the beginning of this experiment the groups were re-equalized by Drs. MacLeod and Woodyard as previously described in order to include newcomers to the orphanage in

the long term observations. Figure 3<sup>5</sup> shows in graphic form the comparative gains in proficiency made by the two groups of fifty-five children each in the selected activities during the

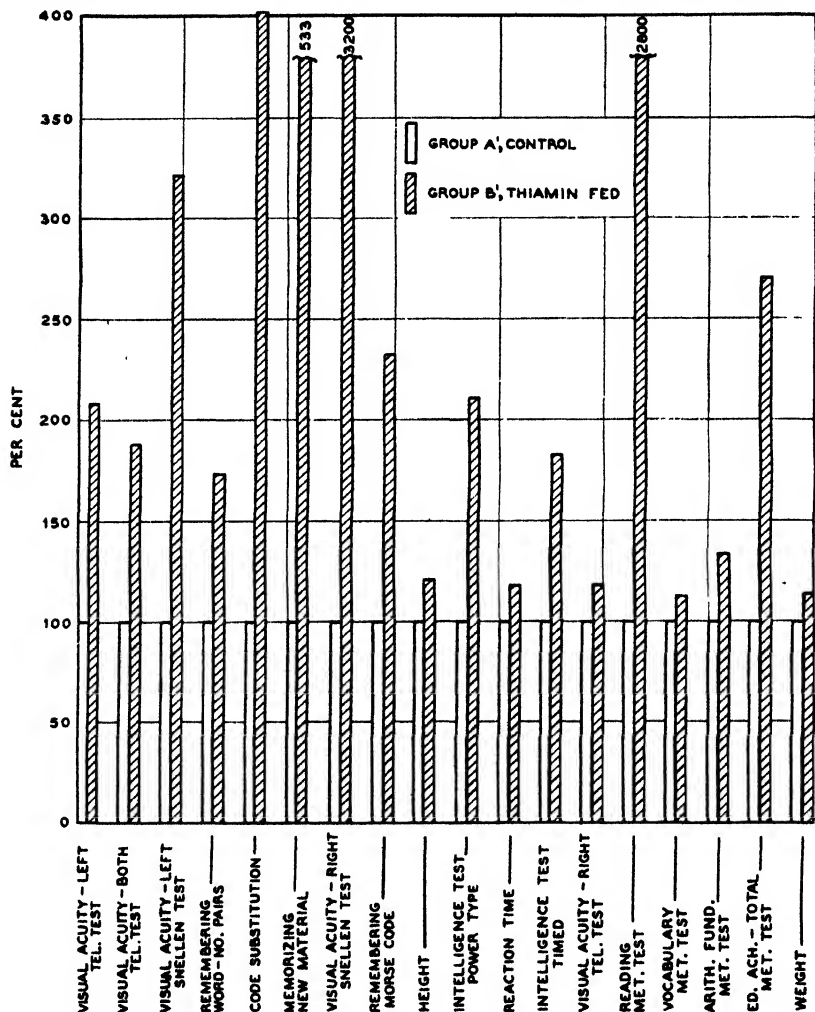


Fig. 3 Relative mean gains made by groups A' and B' in a year, 1942-1943 (letting gain of group A' be 100%).

<sup>5</sup> Tabular data from which figure 3 was drawn are given in the full account, "Further Effects of Added Thiamin on Learning and other Processes," Bureau of Publications, Teachers College, Columbia University, New York.

year. In all fifteen tests the vitamin fed group surpassed the control group in average gain. The *t*-ratios, one for each activity, were computed as before and are given in table 3. Eight of them are so large that assumption of chance is difficult to justify, especially in view of the fact that all the superiorities are in the same direction.

TABLE 3

*Superiority of thiamine fed group B' over control group A' in gain in first year-long experiment, September 1942-September 1943.*

ACTIVITY	NO. OF PAIRS	T-RATIO	PROBABILITY THAT SUPERIOR GAIN WAS DUE TO CHANCE
1. Visual acuity, left eye telebinocular test	40	4.91	.000002
2. Visual acuity, both eyes telebinocular test	40	3.84	.0001
3. Visual acuity, left eye Snellen test	45	3.41	.002
4. Remembering: word-number pairs	20	3.23	.002
5. Code substitution	51	3.20	.003
6. Memorizing new material	47	2.83	.007
7. Visual acuity, right eye Snellen test	45	2.75	.01
8. Remembering: Morse code	20	2.70	.02
9. Height	43	2.28	.03
10. Intelligence power type test	55	2.15	.04
11. Reaction time <sup>1</sup>	52	2.04	.05
12. Intelligence timed test	48	1.85	.07
13. Visual acuity, right eye telebinocular test	40	1.69	.1
14. Educational achievement Metropolitan test, total score	39	1.62	.1
15. Weight	43	.94	.3

<sup>1</sup> Choice Reaction Time device was loaned by the Traffic Engineering and Safety Department, American Automobile Association, Washington, D. C.

*Second year-long experiment, 1943-1944*

With slight changes in the program of tests, the experiment was carried on through a second year after regrouping half the number of children so that of thirty (out of the first year's total of sixty) pairs, the member who had received placebos the first year received thiamine tablets the second year, and the member who had received thiamine the first year received placebos the second year. The purpose of this reversal was to exclude the possibility that the children chosen originally for the thiamine group were accidentally somewhat superior genetically or otherwise. Reversal of the entire number of sixty pairs was avoided. Since it was known at the orphan-

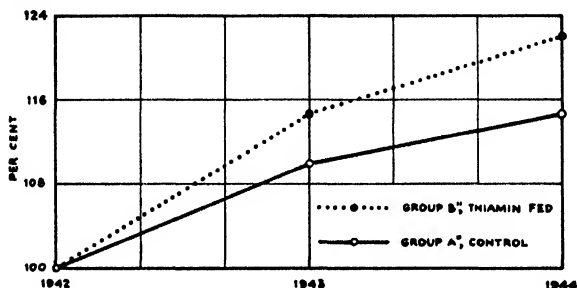


Fig. 4 Average performance of twenty non-reversed pairs in 2 years, eleven activities combined. (Each item is expressed as a percentage of its mean initial score).

age that the children had been arranged in two groups, there was danger that someone would guess a general reversal had occurred and that this would introduce some subjective influence on the results if such a belief became general. Again as in the previous experiments, the full schedule of tests was confined to those pairs of children who could read. There were twenty pairs of children subject to the full schedule of tests which were reversed and twenty pairs which were not reversed, all of whom remained in the institution throughout the 2 years.

The effect of reversal in the second year-long experiment must be considered by comparing the results of the second

year with those of the first. The combined average percentage gains for all eleven activities (not including the memory tests omitted in the measurement of primary grade children) practiced by twenty pairs of children which were not reversed

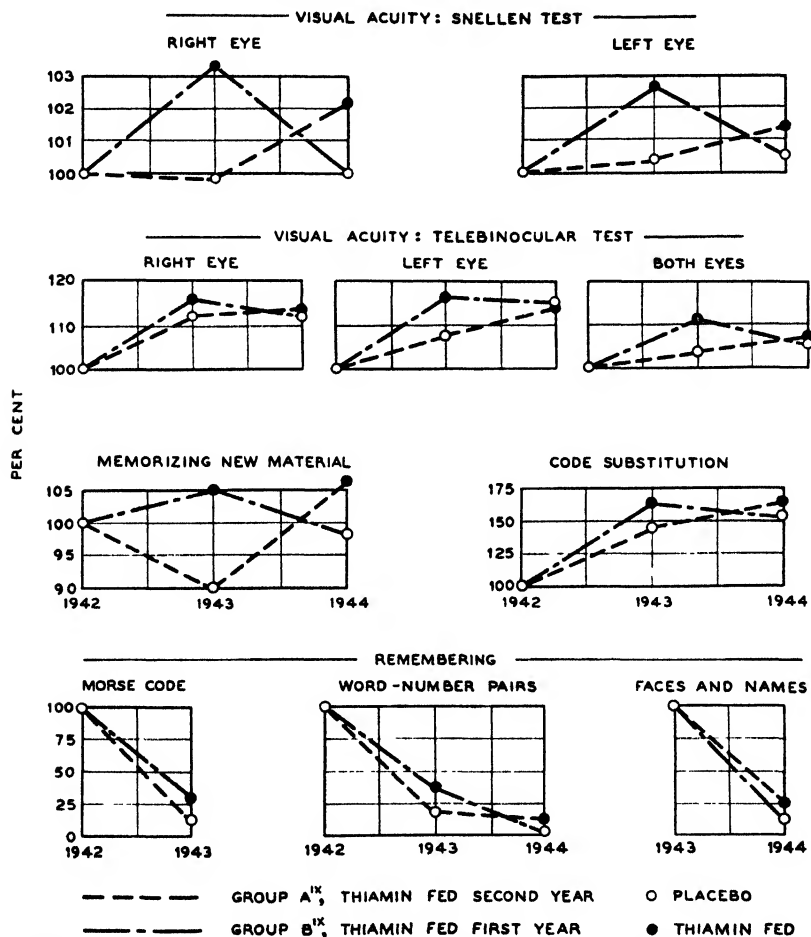


Fig. 5 Performance of twenty reversed pairs showing reversed superiority of gain. (Measures were made at approximately annual intervals).

are shown graphically in figure 4. Trends for the average of each activity separately do not in any case differ greatly from that of the composite shown in figure 4. The vitamin fed children gained an average of 15% in performance the first

year and 22% the second year; the control group gained 10% and 15%, respectively. The reader is referred to the full presentation<sup>1</sup> of the study for further detail which cannot be presented here for lack of space.

Examination of the corresponding tests of the twenty pairs of children whose thiamine supplement was reversed the second year reveals two somewhat different trends. The tests which revealed reversed superiorities of gain are shown in figure 5 and those which do not are presented in figure 6. In

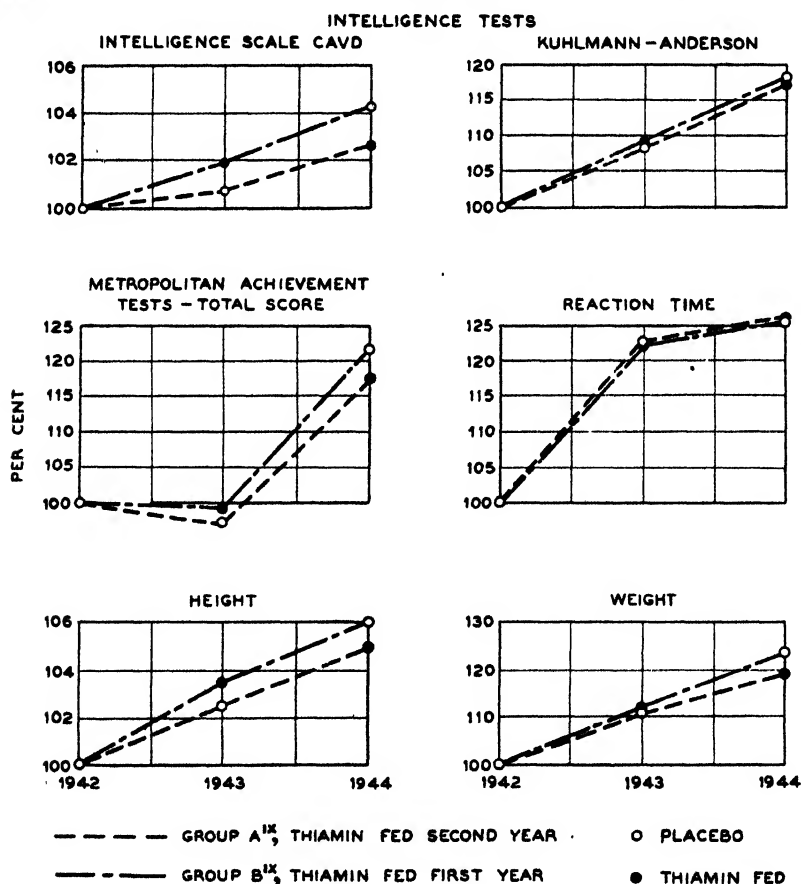


Fig. 6 Performance of twenty reversed pairs in activities not showing reversed superiority of gain. (Measures were made at approximately annual intervals).

the former the gain curves cross following reversal as would be the case if the current intake of thiamine were the controlling factor; in the latter, the curves do not cross. Evidence is wanting of the effect beyond a year's duration of withdrawing thiamine from those who had previously received it. The results suggest that the characteristics referred to in figure 6 change more slowly but more permanently and that the effect of the thiamine taken the previous year held over appreciably into the second year. It is clear that the results of thiamine supplementation are not sufficiently great to be observable over short periods of time with respect to all measures of performance. The cumulative effects throughout a lifetime, however, may nevertheless spell the difference between alert, successful living and a marginal effectiveness.

#### SUMMARY

During three periods of time between May 1941 and September 1944, the effect was observed of daily supplementation of the dietary of an orphanage with 2 mg of thiamine per child.

Thirty-seven to fifty-five carefully matched pairs of children were used, one member of each pair receiving thiamine and the other an indistinguishable placebo. No participant, child or adult, had knowledge concerning the group to which any child belonged.

Measurements of performance included acuity of vision, skills at games, reaction time, reading, arithmetical processes, memorizing and forgetting, intelligence tests and other recognized measures in current use by psychologists. The schedule of tests was adapted to the length and circumstances of each period.

In the first period of 6 weeks the vitamin fed group made superior average gains in performance in every one of the eighteen test tasks. The individual variations in gains within each group were within the limits of probable error in the case of seven of these tasks.



In the second period of 1 year, fifteen activities were used and the vitamin fed group again surpassed the control group in gains in performance in every activity. The superior gain of the vitamin fed group was statistically significant for eight of the fifteen activities.

In the third period of 1 year, without knowledge of the participants twenty pairs of children were continued with the same regimen as in the previous year while twenty pairs were so reversed that those who had received thiamine now received a placebo and vice versa. The unreversed pairs continued to show superiority of average performance for the thiamine fed group in all eleven tests used; the reversed pairs showed reversals of superior gain in seven activities but failed to show adverse effects of withdrawal of thiamine in intelligence tests, educational achievement, reaction time, height or weight gains.

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# STUDIES ON CAROTENOID METABOLISM

## VI. THE RELATIVE PROVITAMIN A ACTIVITY OF CAROTENE WHEN INTRODUCED ORALLY AND PARENTERALLY IN THE RAT <sup>1</sup>

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### TWO FIGURES

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Although there can be no doubt that  $\beta$ -carotene is the mother substance of vitamin A in the animal body, little is known of the mechanism of such a transformation or of the site in the organism where the change may occur. It is generally considered that an enzyme, carotenase, in the liver is responsible for effecting this change but the experimental evidence substantiating such a hypothesis is exceedingly tenuous. Olcott and McCann ('31) presented the first positive results when it was shown that a slight inflection could be detected in the absorption spectrum at 328 m $\mu$  when rat liver slices or liver brei was incubated with a carotene sol for 36 hours. Vitamin A was not isolated, however, and the transformation proceeded only to an extremely limited degree if one is willing to accept such a formation from the spectrophotometric proof offered. In a number of cases there has been a failure to demonstrate any in vitro changes of carotene in the liver. No alterations in this substance could be shown in the liver of the shark (Euler and Euler, '31) or the cat (Rea and Drummond,

<sup>1</sup> The material contained in this paper is part of a thesis submitted by Edwin L. Sexton in partial fulfillment for the degree of Doctor of Philosophy in Biochemistry in the Graduate School of the University of Southern California.

<sup>2</sup> Now with The Best Foods, Inc., Buffalo, New York.

'32; Drummond and McWalter, '33). Pariente and Ralli ('31-'32) have reported one successful experiment out of four attempts where the formation of vitamin A from carotene was demonstrated in dog liver. Euler and Klussman ('32) obtained positive results on cow liver as did Wilson et al. ('37) on rabbit liver undergoing anaerobic autolysis.

Some of the discrepancies between the results of different investigators may be related to species variations in the metabolism of the carotenoids, a variability which has long been recognized (Zechmeister, '37). Jensen and With ('39), for example, have found wide differences in the carotene content in the livers of various species. In an extensive study which included 33 mammals comprising 21 species, 41 birds of 36 different species, 4 reptiles from 2 species and also 8 different human specimens, it was found that carotenoids were completely absent in many of the livers examined. Considerable amounts of these polyenes were found in the livers of beasts of prey, birds and the herbivora. Similar discrepancies in the carotenoid content of the blood and tissue fat between different species also have been noticed. Thus, it is known that these pigments are absent from the blood and tissue fat of the rat and pig, but they may be detected in varying amounts in the case of chickens, cattle, horse and men (Zechmeister, '37).

It has previously been reported from this laboratory that a marked decrease in the carotene excretion in milk occurred as a result of the administration of massive doses of vitamin A to cows (Deuel et al., '41); a simultaneous lowering in blood carotene also occurs (Deuel et al., '42). That this behavior is not confined to the cow was shown by a similar reaction in the hen where the yolk carotenoid as well as that in the liver, blood and in the tissue fat was markedly lowered on diets containing large doses of vitamin A (Deuel et al., '43).

The present experiments were designed to determine whether an increased rate of disappearance of carotene could be shown as a result of an augmentation in the amount of carotenase in the livers of rats previously treated with abnor-

mally high doses of vitamin A. It is also possible that an increased amount of lipoxidase, an enzyme first described by Hauge ('35), might be responsible. Since it was found that parenterally injected carotene remained unchanged in the livers of rats for many days, tests on the comparative biological activity of carotene introduced parenterally and orally were made. As a result certain possibilities regarding the site of carotene conversion to vitamin A have been suggested.

#### METHODS

Rats from our stock colony were kept on a limited intake of vitamin A similar to that employed in preparation for vitamin A bioassays. In the experiments in which the effect of the previous vitamin A intake on the disposal of carotene by the liver was to be followed, weanling rats were divided into five groups and placed on the U.S.P. XII vitamin A-free depletion diet for 28 days. During this interval each group received 0, 30, 450, 900 or 3000 International Units (I.U.) of vitamin A daily which was administered separately from the basal diet. Vitamin A-depleted rats maintained with sufficient vitamin A daily to prevent death but insufficient to clear up the eye symptoms or to produce growth were used for the injection experiments in which the animals were to be sacrificed.

In most cases the carotene employed consisted of 90%  $\beta$ -carotene and 10%  $\alpha$ -carotene supplied by General Biochemicals, Inc. In the bioassay experiments pure  $\beta$ -carotene was used.<sup>3</sup> Liver carotene and vitamin A were determined spectrophotometrically on petroleum ether extracts obtained after a 30-minute hot saponification in the dark in which a total of 25 ml of 95% ethanol was used and also 1 ml of 40% aqueous KOH per gram of liver.

In certain experiments where the liver carotene was characterized chromatographically, the livers were frozen in solid CO<sub>2</sub> immediately on being removed from the animals, and powdered in the frozen state by passing through a Wiley mill. Carotene was extracted with methanol without saponi-

<sup>3</sup> This was kindly furnished us by Prof. L. Zechmeister.

fication from this powder, transferred to petroleum ether and chromatographed on a  $\text{Ca}(\text{OH})_2$  column.<sup>4</sup>

Three carotene preparations were employed. The first sol where carotene was suspended in blood plasma remained perfectly uniform without any loss in carotene when kept for a period of 30 days at 40°C. in the dark. The carotene concentration was adjusted to 60  $\mu\text{g}$ . per milliliter.

A lecithin-stabilized sol was also employed in some of the tests. The carotene and lecithin,<sup>5</sup> dissolved in diethyl ether by shaking, were slowly added to propylene glycol which in turn after evaporation of the ether was mixed with water at 100°C. with vigorous shaking. After standing over night, it was passed through a Buchner funnel. The sol was completely homogeneous and had a concentration of 160  $\mu\text{g}$  per milliliter.

A concentrated carotene solution in cottonseed oil, containing 3000  $\mu\text{g}$ . of carotene per milliliter, was prepared by shaking a diethyl ether solution of carotene with cottonseed oil under nitrogen for 45 minutes, evaporating the ether under reduced pressure and removing undissolved carotene by filtration. The solution was employed in intrasplenic injections. It remained uniform for 3 days or more at room temperature.

The plasma and lecithin sols were well tolerated when introduced orally, intraperitoneally or into the blood stream by cardiac puncture. The oil solution of carotene was satisfactorily administered by intrasplenic injection.<sup>6</sup>

Because of the failure to detect appreciable amounts of carotene in the liver under any conditions where it was fed orally, balance experiments were instituted and the carotene content of the liver was determined after known amounts had been absorbed. In one series of these tests carotene was administered in oil daily over a period of 10 days along with the vitamin A-free diet while in the second series, it was given in natural form as a component of alfalfa flour over a like

<sup>4</sup> We wish to thank Prof. L. Zechmeister for carrying out the chromatographic separation.

<sup>5</sup> Kindly furnished by the American Lecithin Company.

<sup>6</sup> We wish to thank Mr. Frank Cramer who demonstrated this method to us.

period. The latter diet was identical with the vitamin A-free depletion diet except that 25% of the starch was replaced with alfalfa flour. In order to prove that carotene was not destroyed in the gastrointestinal tract, experiments on the excised tract were carried out by a method similar to those on vitamin A reported earlier (Reifman, Hallman and Deuel, '43). In the bioassay tests, rats previously depleted of their vitamin A stores were used.

## RESULTS

*The effect of previous vitamin A feeding on liver carotene following carotene injection*

The carotene and vitamin A concentration in the livers of rats which had previously received various levels of vitamin A daily over a 28-day period following weaning are recorded in table 1 at various periods up to 9 days after the intravenous injection of a carotene sol stabilized in plasma. It should be kept in mind that these and subsequent values for vitamin A

TABLE 1

*The carotene and vitamin A content of the livers of rats at various periods following the intravenous injection of a plasma sol containing carotene after the previous administration of vitamin A at different levels over a 28-day period.*

PERIOD FOLLOWING INJECTION	PER CENT OF INJECTED CAROTENE RECOVERED <sup>1</sup>					LIVER VITAMIN A IN I.U. PER GM				
	1	2	3	4	5	1	2	3	4	5
GROUP <sup>2</sup>										
<i>hours</i>										
0.15	13	22	40	29	32	10	10	396	589	1970
2	68 <sup>3</sup>	60	61	58	73	23 <sup>3</sup>	6	368	528	1410
6	69	75	58	56	67	20	14	362	546	1510
12	67	73	58	59 <sup>3</sup>	54	17	16	198	437 <sup>3</sup>	1670
24	64 <sup>3</sup>	66 <sup>3</sup>	54 <sup>3</sup>	60	61 <sup>3</sup>	18	14 <sup>3</sup>	332 <sup>3</sup>	525	1740 <sup>3</sup>
168	39	42	30	38	35	10	11	297	464	1590
216	34	37	35	32	33	8	11	374	405	2060

<sup>1</sup> The average dose of carotene administered was 105  $\mu$ g.

<sup>2</sup> The previous daily level of vitamin A administration in I.U. was as follows: group 1, 0; group 2, 30; group 3, 450; group 4, 900; group 5, 3000.

<sup>3</sup> Average of two rats. Other results are on single rats.

are not reliable when values of less than about 20 I.U. per gram are involved. The limitations of the method would make it preferable to designate these as "apparent" vitamin A.

It is apparent that no differences obtain over a 9-day period in the rate at which carotene is destroyed in the liver irrespective of the previous level at which vitamin A was administered. It is also evident that no appreciable vitamin A storage took place in the liver when 30 I.U. of vitamin A had been given daily; but with the higher doses the amounts deposited were progressively higher.

*The proof of the purity of the carotene isolated from the liver after carotene injection*

In order to establish the identity of the carotene isolated from the liver with that injected, the absorption curves were determined on extracts of the original plasma sol injected, and on the samples isolated from the livers 7 and 9 days after injection. That the material is entirely unaltered is indicated by the curves given in figure 1. The points for the two maxima are identical in all three samples. The identity of the sample of carotene isolated from the liver was further established as all-trans- $\beta$ -carotene by chromatographic separation.<sup>4</sup>

*The comparative amounts of liver carotene and vitamin A after parenteral and oral administration of carotene*

A further comparison of the effect of injecting carotene by various pathways was carried out on rats previously depleted of vitamin A. Since the livers were already depleted of vitamin A, it was possible to determine whether an accumulation of vitamin A takes place in the liver. These data are summarized in table 2.

Whereas the basal level of liver carotene was practically zero, a considerable amount was found in this organ when carotene was injected by the intravenous or intraperitoneal route as the plasma or lecithin-sols or by the intrasplenic route

in the cottonseed oil solution. However, no measureable amount of the liver carotene was present after the oral administration of the carotene sols. The same was true when a diet containing 25% of alfalfa was fed.

There was no increase in the vitamin A content of the liver after the intrasplenic injection of the carotene in oil solution although this value was markedly increased after carotene or

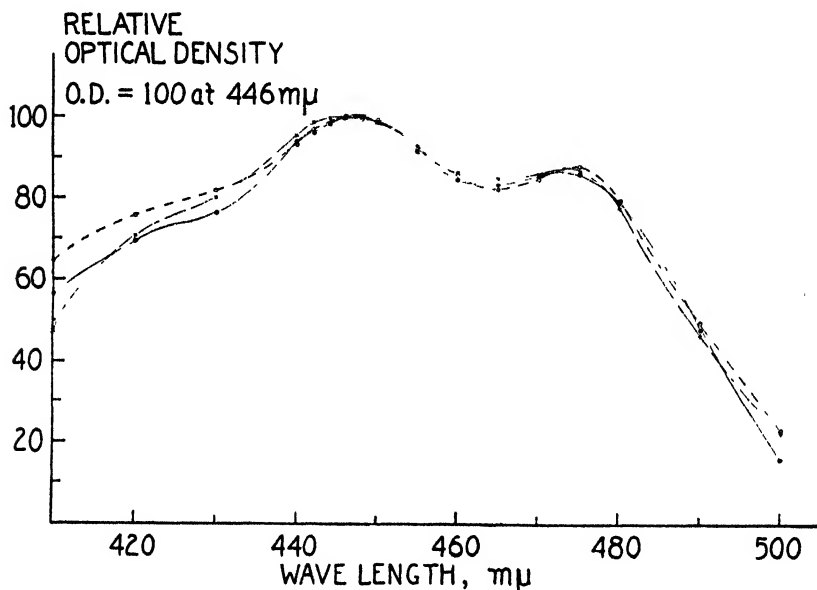


Fig. 1 The absorption curves of carotene extracted from the original sample of plasma-stabilized  $\beta$ -carotene sol (solid line), of carotene isolated from liver after 7 days (dashed line) and 9 days (dashed and dotted lines).

vitamin A was given orally in cottonseed oil or after the alfalfa diet. It seems probable that the low basal levels actually determined for carotene and vitamin A in the liver are within the experimental error of the method or that may represent impurities rather than the actual provitamin and vitamin A. It is probable that these values would be reduced to zero if the liver hydrolysates were purified by chromatographic adsorption.



TABLE 2

*Summary table of the carotene and vitamin A content of the livers of rats previously on a vitamin A-free diet after the administration of carotene by several parenteral pathways, of an alfalfa diet, of vitamin A orally and when no supplement was given.*

MATERIAL ADMINISTERED	SOLVENT	PATH-WAY <sup>1</sup>	NO. OF RATS	QUANTITY ADMINISTERED	ELAPSED PERIOD <sup>2</sup>	LIVER CONTENT	
						Total carotene	Vitamin A
				$\mu g$	hours	$\mu g$	I.U./gm
Carotene	Plasma	O.	2	104	24	5.7	.
			5	1138	24	6.5	.
		IP.	2	104	17	41.6	.
			4	1138	24	176.8	.
	Lecithin	O.	5	1900	24	9.5	...
		IP.	3	316	24	91.3	.
		IV.	4	316	24	189.9	.
	Cotton-seed oil	O.	5	1720	24	6.2	.
			5	2910	24	3.4	13.3
			3	3020 <sup>3</sup>	240	2.9	109.7
		IS.	3	875	24	578	9.1
			5	975	240	529	7.9
Vitamin A	Cotton-seed oil	O.	4	1680 <sup>3,4</sup>	240	.	86.6
Alfalfa diet <sup>5</sup>	.	O.	4	2080 <sup>6</sup>	336	7.9	42.4
Vitamin A-depletion diet	.	O.	19	0	.	1.5 (0-5.1)	10.8 (8.4-12.1)

Where no values are reported, no determinations were made.

<sup>1</sup> O., oral; IP., intraperitoneally; IV., intravenously; IS., intrasplenically.

<sup>2</sup> This is period elapsing from first administration of carotene to the time when animal was sacrificed.

<sup>3</sup> Given in two equal divided doses on 2 successive days.

<sup>4</sup> I.U. of vitamin A administered.

<sup>5</sup> Containing 25% alfalfa. Fed over 14-day period.

<sup>6</sup> Total carotene ingested in 14 days. This averages 148  $\mu g$  daily.

*Experiments on absorption of carotene*

Since there was no evidence for the accumulation of carotene in the liver after the administration of large amounts of the provitamin by mouth, experiments were undertaken to determine the quantity absorbed from the gastrointestinal tract under a variety of conditions. The summary of these data is recorded in table 3.

TABLE 3

*The recovery of carotene from the gastrointestinal tract of rats fed carotene sols, an alfalfa diet, or a control vitamin A-free diet.*

CAROTENE			NO OF RATS	DURATION OF TEST AFTER FIRST CAROTENE ADMINISTRATION	PERIOD OF FASTING		CAROTENE RECOVERED		CAROTENE UNABSORBED
Type of sol	Total fed	Days administered			Before carotene	After carotene	G i tract	Feces	
	$\mu g$			days	days	days	$\mu g$	$\mu g$	%
Oral feeding									
Plasma	104	1	2	1	2	1	20.3	44.2	62.0
	1138	3	5	4	0	0	104	499	53.2
Lecithin	1900	3	5	4	0	0	290	414	37.0
Cottonseed oil	1720	1	2	1	0	1	465	316	45.4
	1720	1	2	1	1	1	467	236	40.9
	1720	1	1	1	2	1	409	272	39.6
	3020	2	3	10	0	0	6.0	1580	52.4
Alfalfa diet	2080	14	4	14	0	0	14.0	924	45.2
Control diet	0	0	5	0	0	0	2.0	9.6	..
Intraperitoneal administration									
Plasma	1138	3	4	4	0	0	48.7	13.8	5.5

Considerable amounts of the carotene fed were still unabsorbed in the g.i. tract 24 hours after the last feeding of the provitamin. Where the feedings of carotene had been continued over several days, a fairly large proportion was lost in the feces. After the administration of 1720  $\mu g$ . in cottonseed oil,

the loss by this route was of the order of 15% ; on the other hand, after flooding the organism with 3020  $\mu\text{g.}$  in cottonseed oil, slightly over 50% was accounted for in the feces over a 10-day period. Since the experiment was continued for 9 days after the last feeding of carotene, it is altogether possible that the carotene may have partially been absorbed and reexcreted. Also, about 45% of the carotene (exclusive of carotenols) fed in the alfalfa diet was excreted in the feces. The control tests on rats fed on a diet, which was carotenoid-free for several weeks, indicates that the quantity of carotene extracted from the gastrointestinal tract and from the feces on a carotene-free diet is inconsequential.

*The stability of carotene in the excised  
gastrointestinal tract*

In order to determine if the relatively large quantities of carotene which disappeared in the previous tests recorded in table 3 actually were absorbed and not simply destroyed in the gastrointestinal tract, tests were made to determine whether any destruction could be noted of carotene kept in the excised gut for 24 hours at 37°C. After introducing 2090  $\mu\text{g.}$  of carotene in 1 ml of cottonseed oil directly into the stomachs of anesthetized rats using a stomach tube, the gastrointestinal tracts were removed intact, the esophagi were ligated, the carotene solution was manually forced throughout the alimentary tract and they were placed in the incubator in glass-stoppered flasks for various intervals. The percentages of the original amounts of carotene administered which were recovered after various periods were as follows: 6 hours, 100 and 101% ; 12 hours, 90 and 102% ; 24 hours, 97, 98, 100, 101%.

These values compare well with experiments with the plasma sol where the extent of the recovery was determined on removing the g.i. tract immediately after the administration of approximately 100  $\mu\text{g.}$  of carotene. The values found in several experiments were 100, 102, 105, 106, and 107%.

*Bioassay experiments*

A series of bioassay tests were made in order to compare the effectiveness of an excess of carotene given orally or intrasplenically and of vitamin A given by the latter route in supplying the vitamin A requirements of vitamin A-depleted rats fed on the U.S.P. XII vitamin A-depletion diet. The supplements were administered in a single dose at the start of the experiments. Five groups of rats were used as follows: group 1, negative controls which received no supplement; group 2, carotene given orally; group 3, carotene given intrasplenically; group 4, vitamin A given intrasplenically; group 5, a continuation of the survivors of group 3, with carotene given orally on the forty-sixth day. Some rats in each group except the negative controls were sacrificed during the course of the experiment to ascertain the level of carotene and vitamin A in the liver. All animals in the negative control group had died before any rats in the other groups were sacrificed. The remaining animals were continued on the vitamin A-free diet until death occurred. The rats were weighed at approximately 5-day intervals. A summary of the data on growth and period of survival is given in table 4.

Although the dosages of the carotene and vitamin A are not identical, they were far in excess of what could be stored and were calculated to give the maximum effect for a single dose. That this supposition is justified is indicated by the identity of the results of groups 2 and 4 where the same maximum increase in weight resulted, the day at which the maximum was reached is practically the same and the period of survival is of the same order. The dose of carotene given orally was higher than that given parenterally to compensate for the carotene lost in the stools in the former case. With the group which received carotene intrasplenically, the maximum weight increase was smaller and occurred on the thirty-fifth day. By the forty-sixth day on which a supplementary dose of carotene was given orally, three of the group had already died and the rest had all lost considerable weight from their

TABLE 4

Summary table showing the maximum gain in weight and average length of life of vitamin A-depleted rats on a vitamin A-free diet after a single dose of carotene or vitamin A.

GROUP NO.	SUPPLEMENT ADMINISTERED	NUMBER OF RATS		AVERAGE DOSE GIVEN	DEPLETION WEIGHT	MAXIMUM INCREASE IN WEIGHT <sup>2</sup>	AVERAGE DAY OF DEATH <sup>3</sup>
		Start	Killed or died before 46 days <sup>4</sup>				

Male rats							
1	Negative control	9	9	0	101.4		19.4(9)
2	Carotene orally	7	2(1)	1480 $\mu$ g	100.4	104.6(50)	66.8(6)
3	Carotene intra-splenically	4	1(2)	592 $\mu$ g	94.0	33.7(35)	*
4	Vitamin A intra-splenically	6	3	415 I.U.	96.8	108.0(50)	84.0(3)
5	Carotene orally	1		1500 $\mu$ g	125 <sup>5</sup>	54 (30)	46 (1)

Female rats							
1	Negative control	6	6	0	103.5		15.3(6)
2	Carotene orally	6	3	1480 $\mu$ g	98.7	72.0(55)	90.3(3)
3	Carotene intra-splenically	9	3(1)	592 $\mu$ g	101.3	36.5(35)	*
4	Vitamin A intra-splenically	8	4	415 I.U.	105.2	74.5(60)	104.5(4)
5	Carotene orally	5		1500 $\mu$ g	148.0 <sup>5</sup>	36.4(44)	82.6(5)

<sup>1</sup> The values in parentheses are the additional number of rats which died before the forty-sixth day.

<sup>2</sup> The figure in parentheses indicates the day on which the maximum increase in weight was reached.

<sup>3</sup> Excluding those killed for the analysis of the livers. The number of rats in the average is included in parentheses.

<sup>4</sup> Continued as group 5.

<sup>5</sup> Because the rats of group 3 which died or had been killed before starting as group 5 had lost more than the survivors, there is an apparent discrepancy between the average maximum weight increase in group 3 and the depletion starting weight of group 5. However, all the survivors in group 3 had started to lose weight before starting in group 5.

maximum levels. Eye symptoms of avitaminosis A had re-appeared.

A comparison of the growth curves is shown in figure 2.

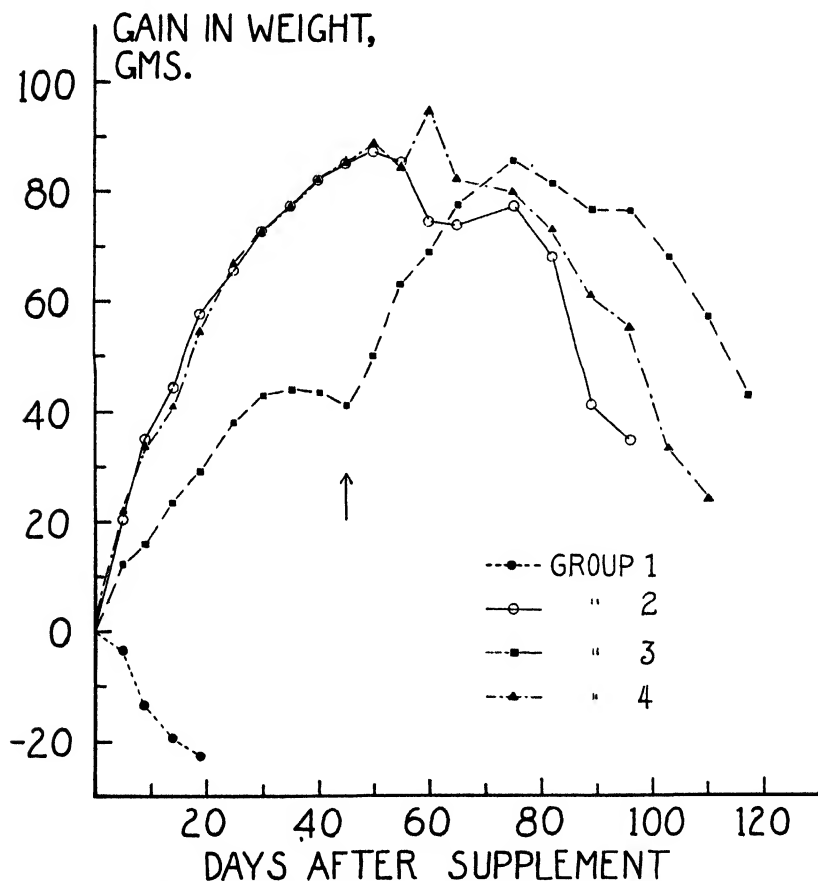


Fig. 2 The gain in weight of vitamin A-depleted rats which received no vitamin A supplement (group 1), or a single supplement at 0 days of carotene orally (group 2), of carotene intrasplenically (group 3), followed by carotene orally at 46 days as indicated by arrow (group 5 only), or vitamin A intrasplenically (group 4).

Table 5 gives the values for liver carotene for the rats which were killed on the forty-sixth day or died earlier in groups 1, 2 and 4; table 6 also gives additional data on group 3.

The carotene isolated from the livers of rats 46 days after the intrasplenic injection was proved to be all-trans- $\beta$ -carotene by its absorption curve and by chromatographic separation.<sup>4</sup>

TABLE 5

*The average carotene and vitamin A in the livers of male and female rats which died before or were killed on the forty-sixth day after the supplements were administered.*

GROUP NO. <sup>1</sup>	AVERAGE DOSE GIVEN	LIVER CAROTENE IN $\mu$ G		LIVER VITAMIN A IN I.U./GM	
		Individual determinations	Average	Individual determinations	Average
1	0	0.4, 0.4, 0.7, 1.1, 1.1, 1.1, 1.4, 1.9, 2.7, 2.7	1.3	6.7, 7.5, 8.0, 8.1, 8.2, 9.5, 9.9, 10.3, 11.4, 12.3, 20.6	10.2
2	1480 $\mu$ g	0.8, 1.9, 2.3, 4.2, 4.6, 6.5	3.4	5.9, 6.5, 10.9, 11.9, 17.8, 30.2	13.9
4	415 I.U.	3.1, 4.2, 4.6, 5.1, 5.1, 5.5	4.9	10.8, 13.5, 15.9, 16.7, 24.3, 25.8, 26.5	19.1

<sup>1</sup> The group numbers are the same as in table 4 and figure 2.

TABLE 6

*Summary of body weight changes and of the carotene and vitamin A in the livers of rats previously injected with carotene intrasplenicly. These died on the forty-fourth day or were killed on the forty-sixth day.*

RAT NO.	SEX	BODY WEIGHT IN GM			LIVER	
		Depletion	Maximum <sup>1</sup>	Final <sup>2</sup>	Carotene	Vitamin A
					$\mu$ p	I.U.
30	M	108	127(25)	112(45)	141	18.4
31	F	92	125(35)	115(45)	171	17.4
33	F	93	135(25)	121(45)	76	12.4
41	F	106	144(25)	126(45)	77	8.3
39	M	90	133(35)	128(40)	185 <sup>3</sup>	41.5 <sup>3</sup>
42	F	119	128(14)	91(40)	214 <sup>3</sup>	98.8 <sup>3</sup>
Average <sup>4</sup>					116	14.1

<sup>1</sup> Figures in parentheses are the first day the maximum weight was attained.

<sup>2</sup> Figures in parentheses are the day of the last weight before the rat died or was killed.

<sup>3</sup> Animals died on forty-fourth day. Because post mortem autolysis of liver had started before the organ was removed, these results are not included in average.

<sup>4</sup> Average of first four rats only.

No appreciable amount of vitamin A over the basal level was found after intrasplenic injection of carotene except in the case of rats 39 and 42 which died of vitamin A deficiency. Had this amount of vitamin A been available earlier, it presumably should have been sufficient to alleviate the deficiency symptoms.

#### DISCUSSION

Carotene was not found in the liver of the rat even after large amounts were administered as the plasma or lecithin sol or as a cottonseed oil solution provided the oral route was used. Moreover, carotene could not be detected in the liver of rats which had received a diet containing 25% of alfalfa flour for 14 days. That the carotene administered orally must have been absorbed in large part was indicated by absorption experiments as well as by the proof that it was not destroyed in the excised gut over a 24-hour period. Further proof that the carotene had been metabolized is afforded by the fact that increased levels of vitamin A were found in the liver over those of the control animals.

On the other hand, carotene is deposited in the liver to a large extent when given parenterally either as a plasma or a lecithin sol or in cottonseed oil solution. Moreover, it is retained in the liver over long periods of time since relatively large amounts of stereochemically pure carotene were found 46 days after the injection.

In spite of the fact that relatively large amounts of carotene may remain in the liver after its parenteral injection, such carotene cannot be used as a source of vitamin A. The livers of two rats which died on the forty-fourth day after intrasplenic injection of carotene had 185 and 214  $\mu\text{g}$  of carotene, respectively. This quantity would be sufficient to cause limited growth over more than a year if administered at a level of 0.5  $\mu\text{g}$  daily as we have obtained a minimum growth for 28 days in bioassay tests at that level (Deuel et al., '45).

When carotene is introduced intrasplenically, it may be argued that it is immediately taken up by the Kupfer cells on reaching the liver where it is retained as any foreign substance



would be (Maximow and Bloom, '42). However, vitamin A when injected by the same pathway is utilized. Under normal conditions when carotene is given orally one should expect it to reach the liver from the gastrointestinal tract in part or at least also by the portal route. Why should it not under these conditions also remain immobilized in the liver?

In the first place, it is possible that it may be metabolized if it reaches the liver in low concentrations while if administered in large amounts, it is deposited in the Kupfer cells and can only with difficulty be utilized if at all. However, the fact that there is no indication of a better utilization when administered in low doses as compared with high concentrations would seem to speak against this idea. Secondly, the possibility exists that carotene may normally be largely absorbed by the lymphatics and then reach the liver first through the hepatic circulation, thus avoiding the portal route. However, in the experiments where the plasma sol was injected into the heart, this pathway should be duplicated and a large proportion of the carotene still remained inactivated in the liver. Finally, carotene might under normal conditions be carried to the liver in small amounts as a protein conjugate. Such a combination apparently exists in the case of some species of animals<sup>7</sup> although it has not been detected in rat blood (Zechmeister, '34).

It is possible that if such a conjugated material is formed, it may be removed by the liver of the rat and converted to vitamin A at a rate greater than that at which it enters the blood stream. None of these possibilities offer an entirely satisfactory explanation of the differences obtained for the behavior of parenterally- and orally-introduced carotene.

The experiments confirm those reported earlier by Lease et al. ('42), who found that the amount needed for growth was ten to one hundred times as great when aqueous carotene sols of oil solutions of carotene were given intraperitoneally

<sup>7</sup> One of us (J.W.M.) has shown in confirmation of other workers that the carotenoids in cow's blood migrate in the Tiselius apparatus with the beta globulin fraction.

or subcutaneously as contrasted with the quantity required when the products were administered orally. Deficiency symptoms and death occurred when considerable amounts of carotene were still in evidence in the liver, in lymph nodes in the peritoneal cavity and at the sites of injection. On the other hand, vitamin A was well utilized when given parenterally.

The present experiments not only confirm Lease et al. ('42) but they extend the observations in demonstrating that the deposited carotene is unaltered and that it is stereochemically identical with the injected product. Our experiments also demonstrate that no fundamental disorder in carotene metabolism develops in rats having these abnormal deposits of carotene. Otherwise, carotene administered orally would not correct the deficiency symptoms and cause a resumption in growth.

A further difference in the behavior of carotene administered parenterally as contrasted with that given orally is that it fails to give rise to any vitamin A in the liver. The average values of 7.9 and 9.1 I.U. obtained for apparent vitamin A per gram of liver are within the range found in the livers of vitamin A-depleted control rats (8.4–12.1). After the oral administration of carotene in oil, a mean of 109.7 I.U. of vitamin A was present while after the alfalfa diet 42.4 I.U. were found per gram of liver. Lease et al. ('42) also were unable to demonstrate vitamin A storage in the liver after prolonged parenteral injection.

It is hard to reconcile the data reported here with the current conceptions of carotene metabolism. If carotene is normally transformed to vitamin A in the liver, then one should be able to demonstrate such an enzymatic system in a more convincing manner than has hitherto been possible. One should also be able to find appreciable amounts of carotene in the blood and definite amounts in the liver unless it is assumed that the enzymatic change is so rapid that it would prevent any accumulation of the provitamin. Finally, the almost complete ineffectiveness of parenterally introduced carotene to effect any prolonged remission of avitaminosis A symptoms even when considerable amounts of pure carotene

are still present in the liver would also argue against the liver as the site of activation of the carotene. Coupled with this observation is the fact that no vitamin A is deposited in the liver after such parenterally introduced carotene while considerable amounts are stored after the oral administration of comparable amounts of the provitamin.

One explanation for these phenomena would be that carotene is transformed into vitamin A in the rat before reaching the blood stream. A possible site for the transformation of carotene to vitamin A might be in the intestinal wall. The change does not take place within the lumen as no destruction was found in carotene present in the excised gut over a 24-hour period at 37°C. Considerable amounts of carotene also accumulate in the wall of the intestine during absorption (Shaw and Deuel, '44) so the transformation there cannot be an immediate one.

In earlier experiments on carotene absorption, a complete absorption of this provitamin was indicated when given in oil solution (Shaw and Deuel, '44). After administration of an average of 3640  $\mu$ g of carotene in oil, 3110  $\mu$ g had disappeared from the lumen of the gut by 18 hours. Over 50% (2040  $\mu$ g) was shown to be present in the wall of the intestine at this time. This was in contrast with the results of Kemmerer and Fraps ('38) and a more recent report of Fraps and Meinke ('45). A possible explanation of these discrepancies is that our earlier tests were carried out with rats previously fasted 2 days or more with the result that no feces were produced or if so only at the start of the test. In the experiments of Fraps, the animals were fed during the absorption tests which resulted in carotene being mechanically carried out. When fed as a component of vegetables, it is also probable that an incomplete digestibility may be ascribed to an incomplete breakdown of the vegetable fibers. In the present tests, considerable portions of carotene were excreted in the feces but this is partly to be ascribed to the fact that the rats were fed instead of fasted previous to and during the carotene absorption. In the series where carotene was given in oil to fasted

rats, less than 15% was excreted in the feces. This incomplete absorption in the present tests may also be because smaller rats were employed than in the former experiments.

#### SUMMARY

1. No relation was found between the rate at which carotene was destroyed after parenteral injection in the rat and the previous level of vitamin A administration. Vitamin A had been given at levels of 0, 30, 450, 900 or 3000 I.U. daily for 28 days previous to the carotene administration.

2. No carotene could be demonstrated in the livers of rats after oral administration of carotene as a plasma or lecithin sol, or in a cottonseed oil solution or after feeding a diet containing 25% of alfalfa for 14 days. Increased levels of vitamin A were observed in the livers under such conditions.

3. Carotene was deposited in the liver after parenteral injection. It was still present in considerable amounts 46 days after the injection and it was shown by spectrophotometric measurements and by chromatographic separation to be all-trans- $\beta$ -carotene.

4. No increase in vitamin A could be demonstrated after the parenteral injection of carotene.

5. When an excess of carotene was administered orally or an excess of vitamin A was injected intrasplenically in a single dose to vitamin A-depleted rats which were then continued on a vitamin A-free diet, identical growth responses were obtained, and the times when the maximum weight was attained were similar. The average length of survival without additional vitamin A was also approximately the same. On the other hand, after a similar excess of carotene was injected intrasplenically, only a slight growth was obtained and the animals died with the livers still containing large amounts of  $\beta$ -carotene. The quantities of carotene still remaining in the liver at the time of death were sufficient to have maintained the animals well over a year if given in divided doses orally.

6. No intrinsic impairment in carotene metabolism was found in rats showing signs of avitaminosis-A after intra-

splenic injection of carotene since they were able to utilize this provitamin when given orally.

7. No carotene was destroyed in the excised gastrointestinal tract of rats kept at 37°C. for 24 hours.

8. The possibility is suggested that the conversion of carotene to vitamin A may be an extra-hepatic function in the rat. The wall of the intestine is suggested as a possible site of such transformation.

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# GROWTH AND FOOD PREFERENCE OF RATS FED A LACTOSE-DRIED MILK RATION CONTAINING BUTTER FAT OR CORN OIL<sup>1, 2</sup>

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## ONE FIGURE

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In a series of investigations on the nutritive value of butter and vegetable fats, Boutwell et al. ('43 a, b; '45) and Geyer et al. ('43) came to the conclusion that when lactose was the sole carbohydrate of the diet butter fat promoted better growth of weanling rats than did vegetable fats. The effect was more pronounced on diets containing high levels of lactose. When other carbohydrates or carbohydrate mixtures were used, this difference was not observed. The inferiority of corn oil in the lactose-containing rations could be reduced by the addition of vitamins of the B-complex. Zialeita and Mitchell ('44), and Deuel et al. ('44, '45) did not find butter fat to cause greater gains in weight than did vegetable fats, but their rations did not contain added lactose.

The possibility that there are differences in the nutritive value of these different fats has created so much interest and the conclusions appear to be in such an unsettled state (Food and Nutrition Board, '43), it was thought desirable to gain further information on the problem. In the present experiments it was decided to employ butter fat and corn oil rations used by investigators working at the University of Wisconsin. A study of food preference also was made.

<sup>1</sup> Contribution no. 303, Department of Chemistry.

<sup>2</sup> Supported in part by a grant from the American Dairy Association.



## METHODS USED

*Growth studies*

The strain of rats and rations selected for these experiments were the same as used in some investigations by Geyer et al. ('43).<sup>3</sup> Except for experiments V-A and V-B, the rats were obtained from Sprague-Dawley, Madison, Wisconsin; 40-45 gm males weaned at 20 days of age were specified. The rats used for experiments V-A and V-B were from the colony at this laboratory, the original stock having come from the above source.

The rations consisted of the following: ether-extracted skim milk powder, 50 parts; lactose (milk sugar, U.S.P. Merck), 20 parts; and either butter fat or corn oil, 30 parts. Minerals were added so that 10 gm of the complete ration contained 1.5 mg of elemental iron, and 0.15 mg each of elemental copper and manganese. To each 100 gm of fat were added, carotene,<sup>4</sup> 2 mg;  $\alpha$ -tocopherol, 8 mg; calciferol,<sup>5</sup> 0.05 mg; and 2-methyl, 1, 4-naphthoquinone, 0.75 mg. Spray-dried skim milk powder was obtained from a plant in Northeastern Kansas. Unsalted, sweet-cream butter was made from cream obtained from the College Dairy; the fat was separated by melting the butter and careful decantation from the water and curd. Corn oil<sup>6</sup> was obtained from a local market.

In order that possible variations in ingredients might be checked, rations of the same composition, prepared at the University of Wisconsin, were used for experiment IV-C.<sup>7</sup>

Weanling rats obtained from the dealer were distributed by weight into the experimental groups; those raised in this laboratory were distributed by litter, sex, and weight. The rats were placed in individual cages with wire floors. Drop-pings and any wasted food fell onto papers placed in pans beneath the cages. The animal room was maintained at 26-27°C. Each rat was provided a little more food than would be

<sup>3</sup> R. K. Boutwell 1944 Personal communication.

<sup>4</sup>  $\beta$ -carotene, 90%;  $\alpha$ -carotene, 10%.

<sup>5</sup> Kindly supplied by E. I. du Pont de Nemours and Company.

<sup>6</sup> Mazola.

<sup>7</sup> We are indebted to R. K. Boutwell for supplying these rations.

consumed before the feeding on the following day. Rations were mixed at not longer than 2-week intervals, kept in tightly-closed jars in the refrigerator, and each day only the feed needed to fill the food cups was removed.

The rats were weighed each week. Records were kept of the weight of ration fed and of any wasted food dropping onto papers beneath the cages. Water was provided *ad libitum*.

### *Food preference*

The rats used for a study of food preference previously had been used on both growth and maze-learning experiments. (A report on the latter is in preparation.) During the maze-learning trials a restricted feeding schedule was followed which allowed continual growth but resulted in the rats being motivated by hunger during the tests. At the completion of these learning trials the rats had been on one of the experimental diets from 7 to 10 weeks. Food preference was tested by placing the rats in the entrance to a short runway, at the opposite end of which were small food cups, one containing the corn oil ration, the other containing the butter fat ration. In experiment II-B the food cups were alike, and their positions were interchanged in a random manner. For the other two experiments the corn oil ration was placed in a black cup and the butter fat ration in a white cup of the same design. The cups were kept in the same positions throughout the experiment to enable the rat to learn the location of the food of its choice, if it had such. Except for the small amount of food obtained from the cups during the experiment, the rats were maintained on the restricted feeding of either the butter fat ration or the corn oil ration used during the maze-learning trials.

Twice the rats were placed in the entrance to the runway and allowed to explore the food cups and become oriented to the new situation. On the following days each rat was given two trials in which it was allowed to select and eat the chosen food; records were kept of the first three choices made. A choice was recorded only when the rat ate some of the food

from the cup; nothing was recorded if the rat merely explored and turned away from the food. When not as many as three choices were made, the food selected and eaten for a period of 15 seconds was recorded as the last choice. After the rats had eaten for 15–20 seconds, they were removed from the runway. It is believed this technique enabled the rat to select freely whichever food appeared the more attractive. The duration of the tests was: experiment II-B, 4 days; experiment III, 6 days; and experiment IV-B, 9 days. In each experiment twelve rats were used, half previously having been on each ration.

## RESULTS

### *Growth studies*

The growth studies are summarized in table 1. In all cases the rats fed the butter fat ration made greater average gains in weight and also ate more of the food. In four experiments, II-B, IV-A, V-A, and V-B, the gains were significant at either the 1% or 5% level of confidence. (t-test, Lindquist, '40). Likewise, the difference in average food consumption was significant for experiments IV-A, V-A, and V-B. An analysis of the average efficiency of conversion of food to body tissue showed that male rats fed for 6 weeks in four different experiments utilize either food for growth to a similar degree of efficiency, all values being between 39.6 and 43.7. Females did not appear to utilize the ration as efficiently as the males, although similar gains were made on both rations. Rats fed for 2½ or 3 weeks utilize the ration more efficiently than when fed for a total of 6 weeks.

Although significant average differences in food consumption were found in only three experiments (probably due to individual differences in rats), the gain in weight made by each individual is related to the intake of either ration. In figure 1 this relationship of food consumed to gain in weight has been shown for all the male rats fed for a total of 6 weeks. A plot of the data for females has not been included; it was similar, except for a small shift in the values due to the smaller gains in weight per gram of food consumed.

Smaller average gains, significant at the 1% level, were made on food obtained from the University of Wisconsin laboratory, but the conversion to body tissue did not differ appreciably from that found in a simultaneous experiment

TABLE 1

*Distribution of the rats, food consumed, gains in weight and average efficiency during growth studies.*

EXPERI- MENT NO.	AGE AT START OF EXPERI- MENT AND SEX	DURA- TION OF EX- PERI- MENT	RATION	RATS ON EACH RATION	AV. WT. AT START	AV GAIN IN WEIGHT	AV WT. OF FOOD CONSUMED	AVERAGE EFFICIENCY (GM GAIN/ GM CON- SUMED) × 100
	days	weeks		no.	gm	gm	gm	
II-B	21 (M)	2½	Corn	6	40	57	110	51.8
			Butter	6	40	66	113	58.5
IV-A	22 (M)	3	Corn	12	42	59	121	48.8
			Butter	12	42	71	144	49.4
II-A	21 (M)	6	Corn	6	40	159	367	43.3
			Butter	6	40	164	376	43.6
IV-B	22 (M)	6	Corn	6	41	153	350	43.7
			Butter	6	42	168	384	43.7
IV-C	22 (M)	6	Corn <sup>1</sup>	6	41	131	307	42.6
			Butter <sup>1</sup>	6	41	135	342	39.6
V-A	21 (M)	6	Corn	15 <sup>2</sup>	40	121	300	40.4
			Butter	15	40	143	332	43.1
V-B	21 (F)	6	Corn	11 <sup>2</sup>	41	105	280	37.5
			Butter	11	42	116	317	36.6

<sup>1</sup> Feed prepared at Wisconsin University.

<sup>2</sup> Litter mates raised at K.S.C.; all other rats from Sprague-Dawley.

(IV-B) using rations prepared in this laboratory. The difference in weight gains indicates a difference in the ration even though prepared according to the same directions. Since the effect was observed on both the corn oil and the butter fat rations, the powdered milk may have been the variable ingredient. However, the fact that the rations prepared in this

laboratory were fresher may have influenced the results obtained. Probably this was not an important factor, since the Wisconsin rations were kept in a refrigerator and rancidity was never detected.

Diarrhea affected many rats, except in experiments V-A and V-B. The condition almost always disappeared in from 5-15 days, generally clearing up a little more rapidly in the

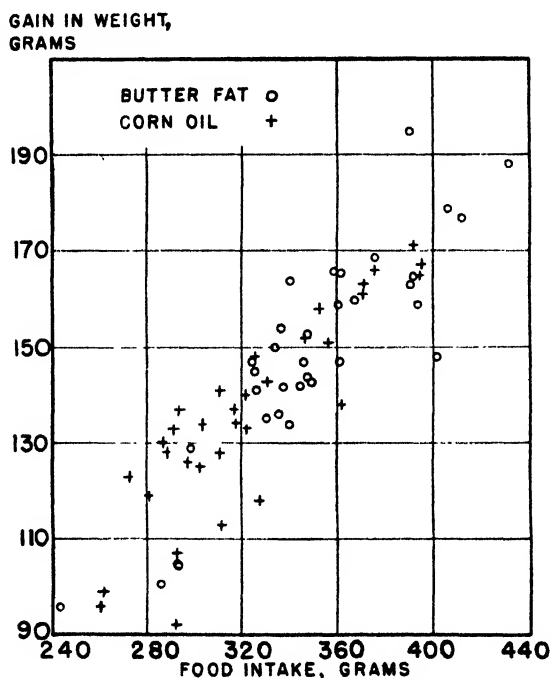


Fig. 1 Scatter diagram showing the relation of gain in weight to food intake for male rats fed butter fat or corn oil rations for 6 weeks.

rats fed the butter fat ration. Rats with diarrhea were not discarded as was done by Deuel et al. ('44), since there is evidence that this condition may possibly be associated with lactose diets (Ershoff and Deuel, '44; Whittier et al., '35). In the entire series of experiments only one animal had to be discarded because of death. This rat appeared entirely normal the previous day. The apparent difference found in the effi-

ency of conversion of the rations to body tissue by rats fed for only 2½ weeks (experiment II-B) may have been due to diarrhea which affected many of the animals. The condition cleared up more rapidly for the rats fed the butter fat ration but remained till near the end of the experiment in rats fed the corn oil ration.

The fur coats of rats on the experimental rations were slightly rougher than those of colony rats fed a complete stock ration, but no differences were apparent among animals on the two experimental rations. A few rats developed a mild alopecia on the posterior part of the abdomen; the condition was not general enough to associate with either diet or experimental group.

### *Food preference*

The first and final (or third) choices of rations in the food-preference experiments are recorded in table 2. In experiments II-B and III the rats showed a tendency to prefer the food previously received; particularly is this true for the rats

TABLE 2  
*Food choice of rats fed butter fat and corn oil rations.*

EXPT. NO.	PREVIOUS RATION	FIRST CHOICE		LAST CHOICE <sup>1</sup>	
		Corn oil	Butter fat	Corn oil	Butter fat
II-B	Corn oil	26	22	28	20
	Butter fat	18	30	14	34
III	Corn oil	34	38	45	27
	Butter fat	15	57	18	54
IV-B	Corn oil	57	51	55	53
	Butter fat	75	33	83	25
Total	Corn oil	117	111	128	100
	Butter fat	108	120	115	113

<sup>1</sup> The food the rat selected and ate for 15 seconds; or if the rat did not eat a selected food for 15 seconds, the third choice was recorded. In some cases the first and last choices are the same, and it is recorded under both headings.

fed the butter fat ration. The results on experiment IV-B are difficult to understand, since the rats previously fed the butter fat ration exhibited a decided preference for corn oil. It is unlikely that anything was seriously wrong with the ration, for the animals previously fed corn oil selected the butter fat ration almost as frequently as they did the corn oil. Furthermore, a new batch of feed was tried with the same results.

While some rats had (or developed) a preference for one or the other ration, learning its location, going immediately to it, and eating it for at least 15 seconds, other rats appeared to be indifferent, sampling first one and then the other ration. A further indication that the rats preferred the food previously received is obtained from the animals showing an exclusive food preference on the last 2 days of the respective experiments. It was found that five "butter" rats preferred the butter fat ration and two the corn oil ration, while seven "corn oil" rats preferred the corn oil ration and three the butter fat ration.

#### DISCUSSION

The fact that in each experiment greater average gains in weight were made on the butter fat rations (significant in four cases) would indicate there is a difference in response when the two fats are incorporated in lactose-skim milk diets. But factors as yet unidentified lead to results which in repeated experimentation are not always reproducible. The differences in intestinal flora and the requirements for the B-vitamins have been suggested as possible causes of differences in response of rats on corn oil and butter fat in rations containing lactose (Boutwell et al., '43; '45). Perhaps variations in the development of the flora, even when rats of the same strain are fed rations made from the same ingredients, are the cause of a variable response from one experiment to another. Comparison of the growth response made on the rations prepared at the University of Wisconsin and the rations prepared in this laboratory indicates another possible reason why results of studies of the nutritive value of fats do not always agree.

A further explanation for the different conclusions which appear in the literature is that no two groups of investigators have published results based on the same experimental rations. It should be noted that these results agree more closely than do any previous studies with those reported by the Wisconsin investigators who used the same strain of rats and high lactose diets.

Although other investigators also have shown that rats consume more of a butter fat ration than of a corn oil ration, opinions differ concerning the reason. Deuel and Movitt ('44) suggest it is due to the fact that rats prefer a butter flavor, while Boutwell et al. ('44) believe the effect is due to a superiority of the ration not associated with flavor.

The technique employed in the present study of food preference differs from other studies in that the rats were motivated by hunger during the tests, but a priori the present technique is not less valid for determining food preference. Although on the experimental diets for 7 to 10 weeks, the hungry rats did not recognize the butter fat ration as superior to the corn oil ration when offered a choice. Likewise, the food containing the natural butter flavor was not selected more frequently by the rats previously fed on corn oil, as would be expected if flavor determined the choice of ration. But a comparison of the results on this study to others wherein ad libitum feeding was practiced brings up a question whether the two rations might appear equal for satisfying immediate needs, although appetite remains longer for butter fat, or this ration produces a physiological condition in the animal which stimulates greater consumption. Thus in ad libitum feeding, this might account for the greater average gains in weight made on certain rations containing butter fat.

#### SUMMARY

1. All groups of rats fed ad libitum on butter fat rations made greater average gains in weight than those fed corn oil rations. The results were significant in four of the seven experiments.



2. All groups of rats fed butter fat showed a greater average consumption of the ration than did those fed corn oil. The results were significant in three of the seven experiments. The gain in weight made on either ration was related to the quantity of food the rat consumed.

3. The average efficiency of conversion of the food to body tissue over a 6-week period was similar for both the butter fat ration and the corn oil ration.

4. When given a choice, hungry rats previously fed corn oil exhibit no preference for the butter fat ration over the corn oil ration. The results in these experiments were not found to be consistent for the rats fed the butter fat ration.

5. Rations of the same composition, made of ingredients from different sources, did not cause the same growth response when used in simultaneous feeding experiments.

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# THE INFLUENCE OF CHOLINE AND OF TRY PAN BLUE UPON THE UTILIZATION OF CAROTENE AND VITAMIN A FOR LIVER STORAGE OF VITAMIN A <sup>1</sup>

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## ONE FIGURE

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According to Thorbjarnarson and Drummond ('38) the storage of fat or cholesterol in the liver tends to increase the total amount of vitamin A stored. When choline was included in the diet the amount of fat in the liver and the amount of vitamin A stored was decreased. In rats allowed to accumulate large reserves of vitamin A by dosing with a concentrate, and subsequently restricted to various modifications of a diet deficient in vitamin A, rapid loss of the vitamin from the liver was observed. The inclusion of a high proportion of fat appeared, however, to retard the rate of loss, this effect again being counteracted by the addition of choline. Very small amounts of vitamin were present in the livers of the rats in the series in which fatty livers were produced by high-fat and cholesterol-containing diets. In the series in which large doses of vitamin A were given, fatty livers were not produced except for mildly fat livers in one group. The data do not indicate that the rate of accumulation or depletion of liver vitamin A was dependent upon the level of fat in the liver although some relation to level of dietary fat was indicated.

<sup>1</sup> This study was supported by a grant from Swift and Co., Chicago.

Lease and Steenbock ('39) compared choline-low high-fat and low-fat diets containing adequate protein, and the same high-fat diet with and without choline, in similar depletion experiments. They found no differences in rate of vitamin A loss due to dietary or liver fat levels.

Popper and Chinn ('42) used the fluorescence microscope to study the distribution of the decreased amount of vitamin A which they found in the livers of choline-deficient rats fed carotene. Chemical analysis showed that these rats had from 1.3 to 19 I.U. vitamin A per gram of liver, in contrast with the normal value of more than 200 I.U. They stated that inability of the liver to convert carotene into vitamin A was not a major factor in causing this decrease since preliminary experiments had shown the same results when vitamin A replaced the carotene. No data on the vitamin A fed rats were given. Clayton and Baumann ('44) measured the depletion of vitamin A in the liver with and without choline using adult rats and mice which had initial uniform stores of vitamin A. They found no difference in the amount of hepatic vitamin A accumulated or retained on the high-fat diets regardless of their choline content or the amount of fat in the livers. In one series they used an adequate protein or methionine-low low-fat diet without choline upon which severe choline deficiency rapidly developed. In 10 or 20 days no difference in vitamin A content of livers, kidneys or other organs developed between the choline-fed and deficient groups. The fat content of the livers was not specified.

In view of these differences it was thought that possibly inability of the liver to convert carotene to vitamin A during choline deficiency might be a more important factor than Popper and Chinn suspected, since they found greater differences in the amount of vitamin A stored during choline deficiency than did the other workers who fed vitamin A rather than the provitamin. Furthermore, since the excess fat in the liver of rats on choline-deficient diets accumulates in the hepatic cells, if less vitamin A were stored by the choline-deficient rat than by the choline-fed rat receiving carotene

this might be indicative of the site of conversion of carotene to vitamin A.

Lasch and Roller ('36) in another attempt to discover the site of storage of vitamin A made a study of the role of the hepatic reticulo-endothelial system. This was done by injecting substances which were taken up by the reticulo-endothelial system in amounts just below the lethal dose, for 3 days prior to, and 3 days during, the injection of vitamin A.<sup>2</sup> Guinea pigs and rabbits whose reticulo-endothelial system had been blocked in this manner had a decreased storage capacity for vitamin A and in a few cases the animals' storage capacity was said to have been abolished completely. On the basis of the results reported the authors concluded that the storage of vitamin A in the liver depends on its reticulo-endothelial system (the Kupffer cells).

With these results in mind an attempt was made to determine the role of the Kupffer cells in the conversion of carotene to vitamin A by feeding carotene to rats whose reticulo-endothelial system had been blocked in a similar manner. Trypan blue was the only dye used in this study, and vitamin A storage was determined after the feeding of vitamin A or carotene.

Thus by packing the hepatic cells with fat by means of choline-deficient diets and the Kupffer cells with the dye, an effort was made to establish in one or the other the site of carotene transformation and of vitamin A storage.

#### METHODS

*Chemical.* The livers were analyzed for vitamin A by a modification of the Davies ('33) method, the Carr-Price blue color being read in the Evelyn photoelectric colorimeter. Total solids were determined by drying the samples in a vacuum oven at 50°C. Liver "fat," was determined by extracting the dried samples with anhydrous ethyl ether in a modified Soxhlet apparatus and weighing the "fat," which

<sup>2</sup> Vogan.

had been dried in a vacuum dessicator after evaporation of the ether.

#### THE CHOLINE EXPERIMENT

*The low-fat diet.* Rats from the stock colony were prepared as for a vitamin A assay, that is, the females were placed on a vitamin A-low skim milk diet for the last week of lactation. The weanling rats were continued on this diet to the twenty-eighth day at which time they had attained an average weight of 57 gm. They were then placed on the low-fat diet employed by Clayton and Baumann ('44) which contained hot-alcohol extracted casein 3.5, cystine 0.15, brewers yeast 3.5, salts <sup>3</sup> 2.9, cottonseed oil 1.5; and dextrin 88.5. Choline was added as 0.3% of the diet fed the control groups. The animals received the experimental diet, which was vitamin A-free, for 1 week without any additional vitamin A, after which the vitamin A <sup>4</sup> or carotene <sup>5</sup> oil supplement was given for 1 month at which time they were sacrificed. The oil supplement was given three times weekly by stomach tube and the other vitamin supplements were injected six times weekly, at a level of 20 µg daily of thiamine, riboflavin and pyridoxine, and 100 µg daily of Ca pantothenate and nicotinic acid amide. The B vitamins were given to supplement the somewhat inadequate amount of yeast in the diet. The litters were evenly divided into groups of ten or more rats of each sex. One group of male and one group of females rats fed carotene also received 3 mg mixed tocopherols <sup>6</sup> daily. The livers from each group of rats were pooled for chemical analysis. Some rats from each group were sacrificed in order to confirm the absence of vitamin A in the liver before the oil supplements were given.

*The high-fat diets.* Similarly prepared rats were placed on a diet containing hot-alcohol-extracted casein 10, fat 35,

<sup>3</sup> Hubbell, Mendel and Wakeman '37.

<sup>4</sup> The vitamin A was given as reference cod liver oil, 0.42 gm total per week.

<sup>5</sup> Carotene in oil concentrate, 2.2 mg per gram. The total amount of oil given was 0.8 gm per week.

<sup>6</sup> Concentrate of natural mixed tocopherols, 40%. This was kindly supplied by K. Hickman of Distillation Products, Inc., Rochester, N. Y.

sucrose 51, and salts<sup>3</sup> 4, when the males weighed approximately 98 and the females 88 gm. The B vitamin supplements were given as in the low-fat diet. Choline was again added as 0.3% of the diet for half the groups. The same oil supplements used with the low-fat diet were given after the animals had been on the high-fat diets for 10 to 12 days. The groups which were to receive carotene plus tocopherols were omitted and the diet was prepared with two different types of fat instead, lard and hydrogenated cottonseed oil. Since lard, commonly used in high fat diets, is known to be deficient in vitamin E, and is highly oxidative, the hydrogenated cottonseed oil which contains tocopherols was employed for half of the animals.

*Preliminary vitamin A analysis of livers.* Seven rats died as a result of choline injection when they were first placed on the low-fat diet. The injection of choline was then discontinued and it was incorporated in the diet. The pooled livers of these weanling rats were found to contain 2.6  $\mu\text{g}$  vitamin A per gram. The livers of twelve rats sacrificed after 1 week on the low-fat diet without any vitamin A supplement were tested individually for vitamin A and all were found to be negative. One rat that received carotene and tocopherols in addition to the choline-deficient diet, killed on the second day was found to have 7  $\mu\text{g}$  vitamin A in its liver. Two similarly treated rats had 6 and 13  $\mu\text{g}$  vitamin A per liver after 20 days of supplementary feeding, while a rat that received choline and vitamin A for the same period had 44  $\mu\text{g}$  of the vitamin in its liver. The livers of three rats receiving the high-fat diet for 1 week without the vitamin were found to be devoid of vitamin A.

## RESULTS

*Changes in body weight.* The rats on the low-fat diet deficient in choline gained twice as much weight as did those which received choline. The reverse was true of the groups fed the high-fat diets. Growth on none of these diets was



normal either with or without choline, but the high-fat diets were superior to the low-fat diet.

*Mortality.* All rats on the low-fat diet survived with the exception of the animals accidentally killed. There were no accidental casualties among the animals on the high-fat diets, but three rats on each type of fat-diet deficient in choline died before the termination of the experiment.

*The fat content of the livers.* When choline was withdrawn from the diet fatty livers resulted in all cases, the effect being greatest on the lard diet and least on the low-fat diet. As the fat content and total weight of the livers increased, the moisture content decreased. When choline was given the total weight, fat and moisture of the livers were normal.

*Vitamin A storage in the liver.* Interpretation of the data involved comparisons of amounts of vitamin A per gram of fresh liver tissue, per gram of non-fat fresh liver, per gram of non-fat liver solids, per gram of liver fat, and per liver. The ability of the active liver tissue to accumulate the vitamin was probably best represented by the vitamin concentration of the non-fat liver solids, or by the total amount of the vitamin retained per liver, since these measures eliminate the influence of the liver fat upon the apparent vitamin concentration. The data are presented in table 1 and the total vitamin A per liver and the concentration of fat in the livers compared in figure 1. Since sex and the addition of tocopherols in some of the carotene groups did not influence the results significantly, all groups on the same diets and receiving the same vitamin supplement were combined in the figure.

On all diets very low stores were produced on 250  $\mu$ g carotene daily, 50  $\mu$ g per liver being the maximum. On the low-fat diet with choline this storage amounted to about one-third of that acquired from 100 I.U. vitamin A daily; without choline it approximated that produced by the vitamin A dosage. On the high-fat diets liver vitamin A from carotene feeding was less than a tenth of the amount produced by vitamin A feeding and was practically negligible.

TABLE 1  
*The storage of vitamin A in 28 days as influenced by the addition of choline to low-fat and high-fat diets.*

DIET	SOURCE OF VITAMIN PER DAY	SEX	NUMBER OF ANIMALS	AVERAGE OF WEIGHT GAIN	LIVER			LIVER VITAMIN A		
					Weight gm	Fat %	Solids %	Per gm	Per liver	Per gm non-fat solids
Low-fat with choline	100 I.U. vitamin A	♂	9	2	2.9	4.1	29	32	93	128
		♀	9	8	2.8	3.9	29	68	190	272
	250 µg carotene	♂	8	4	2.8	3.7	29	13	36	52
		♀	9	3	2.8	3.7	30	18	50	69
250 µg carotene with tocopherols		♂	6	9	3.3	3.8	30	8	26	31
		♀	11	5	2.7	4.2	31	16	43	59
Low-fat with- out choline	100 I.U. vitamin A	♂	12	10	3.3	18.1	39	12	40	57
		♀	11	6	2.9	20.7	41	23	67	115
	250 µg carotene	♂	11	14	3.0	14.0	37	13	39	56
		♀	7	14	3.0	13.8	38	7	21	29
250 µg carotene with tocopherols		♂	10	18	3.6	11.4	39	7	25	25
		♀	9	15	3.2	13.2	38	10	32	40
Lard with choline	100 I.U. vitamin A	♂	7	35	6.0	3.8	30	28	168	108
		♀	6	32	4.8	3.7	30	32	153	123
	250 µg carotene	♂	8	31	5.3	4.7	30	2	10	8
		♀	7	26	4.6	3.5	30	3	13	11
Lard with- out choline	100 I.U. vitamin A	♂	8	17	11.1	34.4	48	13	145	95
		♀	7	16	8.7	44.1	57	21	182	154
	250 µg carotene	♂	8	22	10.8	36.8	51	0	0	0
		♀	6	20	9.4	39.1	54	0	0	0
Hydrogenated cottonseed oil with choline	100 I.U. vitamin A	♂	8	29	5.6	3.0	30	39	218	144
		♀	6	26	4.3	2.9	29	52	223	200
	250 µg carotene	♂	8	35	6.1	2.4	29	2	12	8
		♀	6	26	4.5	3.1	29	6	27	23
Hydrogenated cottonseed oil without choline	100 I.U. vitamin A	♂	6	7	8.9	34.1	49	27	240	180
		♀	6	11	8.5	32.4	49	33	280	250
	250 µg carotene	♂	6	22	9.8	33.6	50	1	10	6
		♀	6	7	8.7	33.6	50	1	9	6

*On the low-fat diet.* On the low-fat diet where fat deposition was relatively low, when choline was withheld the rats receiving vitamin A stored less than one-half as much as did those which received choline, compared on any of the bases available (table 1). When carotene was given, the amount of vitamin A stored was not influenced by the withdrawal of choline. There was in the latter case, some reduction in the amount of vitamin A stored by the female animals but

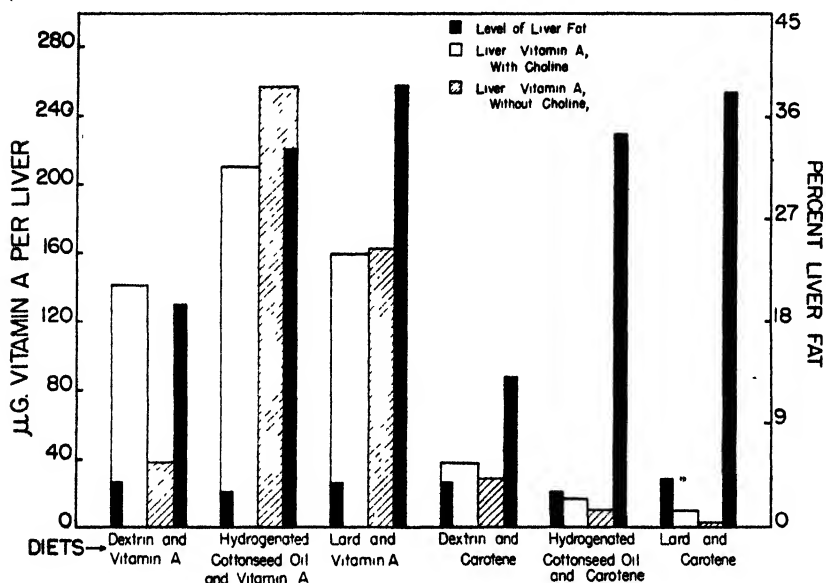


Fig. 1 Total vitamin A and per cent fat in the livers of rats fed dextrin (low fat) and high fat diets with and without choline.

this was not considered to be significant. The amount of vitamin A stored was not influenced by feeding tocopherols with the carotene in this experiment.

*On the high fat diets.* On the lard diet where fat deposition was the greatest, the total amount of vitamin A stored in the livers, as well as the concentration per gram of fat-free liver or liver solids, was nearly the same with and without choline when vitamin A was fed. The concentration of the vitamin in the fresh liver tissue was reduced in the latter case due to

the increased size of the fatty livers (table 1). This was also true on the hydrogenated cottonseed oil diet but in this case the withdrawal of choline resulted in a small increase of doubtful significance in the total amount of vitamin A stored. The accumulation of the vitamin appeared to be consistently greater on the cottonseed oil than on the lard.

When carotene was fed the animals on the high-fat diets stored less vitamin A than did those on the low-fat diet. The amount of storage was less when choline was withdrawn from these diets but it is not likely that this lowering was significant at these extremely low levels.

*Sex.* There seemed to be no clearly established pattern of response which could be attributed to a difference in sex, although in most of the groups the females stored more vitamin A than did the males.

#### CHOLINE EXPERIMENT

##### *Discussion*

*Vitamin A.* The increase in the fat content of the liver induced by a dietary deficiency of choline was associated with a decrease in the vitamin A deposit in the liver of rats receiving a fixed amount of vitamin A with the low-protein, low-fat, high carbohydrate diet. On the low-protein, high-fat diets no such effect was seen. Liver fat deposition was least on the low-fat choline-free diet, hence if vitamin A storage were inversely related to this factor the least effect of the choline should have occurred in this case. But definite improvement in storage with choline was seen only on this diet.

*Carotene.* Differences in deposition of vitamin A in the liver due to choline feeding were small on both types of diet when carotene was fed. In general there was less vitamin A in the liver when choline was absent from the diet but the differences were so small as to be of doubtful significance. In this experiment carotene utilization was inferior on the high fat diets. The large amount of fat in the diet may have reduced the absorption of carotene or the greater weight gains

of these groups may have involved more utilization of the vitamin. However, a similar difference due to growth in the stores of vitamin A fed as such was not observed.

Lease and Steenbock ('39), Clayton and Baumann ('44) and Thorbjarnarson and Drummond ('38) noted the rate of depletion of vitamin A stores on high and low-fat diets or in choline deficiency. In this study the accumulation of such stores on uniform intake was observed.

Lease and Steenbock noted no effect on depletion on choline-low high-fat and low-fat diets nor on high-fat diets with and without choline. Our findings are similar. The effects of low-fat diets with and without choline were not compared by Lease and Steenbock. Clayton and Baumann used both high-fat and low-fat diets with and without choline and detected no difference in rate of depletion of liver vitamin A. In one series of rats on low-methionine low-fat diet for 10 or 20 days substantially the same vitamin A retention appeared to have occurred with and without choline. This is not in accord with our results, but the basal diet used was different from any which we employed. Our findings accord however with the observation of Thorbjarnarson and Drummond that high-fat diets retarded the loss of liver vitamin A, but not in proportion to the amount of fat in the livers. The marked influence of choline on carotene utilization noted by Popper and Chinn ('42) was not seen in this experiment and this lack of confirmation may have been due to the type of diet employed by them. It appears that the character of the basal diet is more important in the accumulation of hepatic vitamin A than the presence or absence of choline or fatty livers. This may also be true of the depletion of these stores.

Apparently whatever may be the mechanisms controlling the utilization of vitamin A and carotene, they are distinct and are not affected by the same condition.

#### THE TRYPAN BLUE EXPERIMENT

The male rats from the litters of four stock-colony rats were carried to weaning in the manner described for the

choline experiments. They were then weaned at 21 days of age directly to a vitamin A-deficient diet made up of casein (alcohol extracted) 22 parts, hydrogenated cottonseed oil 5, brewery yeast 10, cornstarch 60.5, and salts<sup>3</sup> 2.5. The animals were allowed to continue on this diet until their weight curves began to plateau, indicating the onset of vitamin A deficiency. Trypan blue in 1% solution was injected for at least 2 days before any vitamin feeding, and by this time the dye had turned the skin a bright blue. The original plan was to inject 0.5 ml for 5 days followed by a 2-day rest period. Due to evidence of toxicity the number of injections was reduced to 3 in the second and third week, but the full five injections were given during the first and final weeks. Comparable groups of animals selected from the same litters were injected with distilled water. In each case half of the animals received 0.5 mg carotene daily in 0.23 gm oil and half received 60 I.U. vitamin A daily as 0.036 gm reference (II) cod liver oil. The oil supplements were given 6 hours after the injection of the dye and none was given during the 2-day rest period. After receiving this treatment for a period of 4 weeks the animals were sacrificed and the individual livers were analyzed chemically for vitamin A.

### *Results*

There was no decrease in the amount of vitamin A stored by the dye-treated rats when the vitamin itself was fed, although the growth was depressed. Two of the animals had more liver-vitamin A than did animals which had been injected with water, (table 2). When carotene was fed the animals surviving the injection showed depressed growth and somewhat smaller liver stores than their water-injected littermates.

Where the animal failed to survive the trypan blue injections the liver stores were lower regardless of whether the vitamin or the pro-vitamin had been given. This may have been due either to the shorter period of vitamin feeding or to

TABLE 2  
*The effect of trypan blue injections on rats depleted of vitamin A and then fed carotene or vitamin A.*

RAT NUMBER	TRYPAN BLUE 1% SOL. INJECTED	SUPPLEMENT GIVEN		PERIOD OF VITAMIN FEEDING	LIVER VITAMIN A		GROWTH				FINAL WEIGHT	DEFICIENCY SYMPTOMS
		Vitamin A	Carotene		Per gm	Total	Weight gain per week					
							1st	2nd	3rd	4th		
	ml	I.U.	mg	days	μg	μg	gm	gm	gm	gm	gm	
17	0.5	1680		28	3.7	44	4	0	35	33	194	
19	0.5	1680		28	3.5	36	-3	9	24	39	173	
23	0.5	1680		28	trace	trace	12	-24	25	6	122	+
28	0.5	1680		28	2.5	60	9	2	30	28	160	+
27	0.5	1440		24 <sup>1</sup>	trace	trace	19	15	41	..	203	+
13	0.5	540		9 <sup>1</sup>	trace	trace	-4	..	..	..	86	+
				average	1.6	23						
14	..	1680		28	2.9	32	32	38	37	4	236	
20	..	1680		28	4.5	40	29	32	38	-4	192	
25	..	1680		28	1.6	21	43	48	41	31	286	
29	..	1680		28	2.6	27	33	38	35	20	227	
				average	2.9	29						
15	0.5		14.0	28	21	181	24	-6	6	24	170	+
21	0.5		14.0	28	13	105	15	10	-3	21	152	+
24	0.5		14.0	28	18	171	-2	8	1	30	154	+
30	0.5		5.5	11 <sup>1</sup>	10	70	12	..	..	..	133	
16	0.5		4.5	9 <sup>1</sup>	8.9	40	-4	..	..	..	92	
31	0.5		4.5	9 <sup>1</sup>	5.6	35	1	..	..	..	99	
				average	12.8	100						
22			14.0	28	23	203	19	18	24	27	205	
18			14.0	28	22	243	23	19	28	32	226	
26			14.0	28	18	184	32	22	36	22	232	
32			14.0	28	21	177	22	32	21	21	196	
33			14.0	28	17	170	32	23	28	22	225	
				average	20	195						

<sup>1</sup> The animal died before the termination of the experiment.

the occurrence of an initial interference with storage followed by the setting up of a compensatory mechanism. The latter possibility is further indicated since during the early part of the supplementary feeding the injected rats, particularly those receiving vitamin A, failed to gain and several developed symptoms of the deficiency.

### *Discussion*

The decreased storage of vitamin A observed by Lasch and Roller ('36) in guinea pigs and rabbits was not observed in this experiment with rats when the animals were injected with trypan blue. The total storage of vitamin A by the dye-injected rats that had been fed carotene was lower than that seen in the water-injected animals. It is doubtful, however, whether this can be considered significant inhibition of carotene conversion. Examination of the livers of animals sacrificed during the first and second week of dye injection might have indicated more definite differences. There is some indication of an "all or none" phenomenon in the rats fed vitamin A, since the stores of the vitamin in the livers of the dye-treated animals were either normal or negligible. The carotene feeding produced more graduated differences. This may point to different mechanisms for transformation and storage in the liver.

### SUMMARY

The deposition of vitamin A in the livers of depleted rats fed fixed amounts of vitamin A along with high-fat low protein diets was not affected by the presence of excess liver fat or choline deficiency. With a low-fat low-protein basal diet the addition of choline increased the liver vitamin A.

When carotene was fed with the high-fat diets the liver vitamin deposits were very small and were somewhat less in the fatty than in the normal livers. With the low-fat diet the carotene produced better vitamin deposition, which was little affected by the presence or absence of choline.



The hepatic accumulation of vitamin A was much the same when the vitamin was fed with the high-fat and low-fat diets, regardless of liver fat, except for a slight decrease on the lard diet. When carotene was fed there was a definite depression of vitamin A storage on the high-fat as compared with the low-fat diets.

The injection of trypan blue produced no effect upon the deposition of vitamin A in the livers of rats fed a fixed amount of the vitamin. A depression of doubtful significance occurred in the deposits found in similar animals fed relatively large amounts of carotene.

It is concluded that the mechanisms of utilization and storage of vitamin A and carotene are probably affected by different conditions and that the composition of the accompanying vitamin-deficient basal diets is an important factor in determining their efficiency.

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# THE NUTRITIVE VALUE OF CANNED FOODS

## XVII. PYRIDOXINE, BIOTIN AND "FOLIC ACID"<sup>1</sup>

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Since the National Canner's Association-Can Manufacturer's Institute Nutrition Program was inaugurated 3 years ago, a great many values for the vitamin A, carotene, thiamine, niacin, riboflavin, pantothenic acid, and ascorbic acid content of canned foods have been obtained (Ives et al., '44, '45; Pressley et al., '44; Thompson et al., '44). After study of the older, better-known vitamins had been completed, it was decided to conduct a survey of canned foods to determine the content of some of the newer B-complex factors: pyridoxine, biotin and "folic acid."

### EXPERIMENTAL PROCEDURE

The method of collection of the samples was essentially the same as that described by Clifcorn ('44). Approximately ten samples of each of the following products were assayed for the factors under consideration: green asparagus, carrots, green beans, yellow corn, grapefruit juice, peaches, peas, salmon, spinach and tomatoes. The samples were assayed within 6 weeks of the time of their arrival and were stored at room temperature during that period.

<sup>1</sup> Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

<sup>2</sup> Now at Research Department, American Can Company, Maywood, Illinois.

### *Preparation of samples*

A sample for analysis consisted of six consumer size cans or one to three no. 10 cans. The solids and liquids were separated by the use of a drained solids screen. The solids were weighed, and the liquid volume determined. In order to determine the distribution of nutrients between the solid and liquid portions of the can, the solids and liquid portion of five samples of each product were analyzed separately. In these instances 100 gm aliquots of the combined solids of a sample were blended in a Waring blender with 100 ml of water and 2 to 3 ml of chloroform. Chloroform was added directly to the 100 ml aliquots of the combined liquids and to all of the grapefruit juice samples. For the five remaining samples of each product, one-tenth of the solid and liquid portion samples were recombined and this reconstituted aliquot blended with 40 to 3 ml of chloroform. The brine was discarded from all of the salmon samples, and they were treated in the same manner as the solid aliquots.

### *Assay methods*

*Pyridoxine.* Samples were prepared and assayed according to the procedure of Atkins et al. ('43). *Saccharomyces carlsbergensis*, the organism employed, responds approximately equally to pyridoxine, pyridoxamine, and pyridoxal (Snell and Rannefeld, '45). Duplicate assays were set up on different days.

*Biotin.* The samples were assayed according to the procedure of Shull, Hutchings and Peterson ('42) using the modification of the medium reported by Shull and Peterson ('43). The samples were hydrolyzed for assay by autoclaving with 4 N sulfuric acid for 2 hours at 15 pounds pressure.

*"Folic acid."* The samples were analyzed for both the *S. lactis* activity and the *L. casei* activity against a standard of crystalline vitamin B<sub>12</sub>. The *S. lactis* factor was measured by the method of Luckey, Briggs and Elvehjem ('44). The titrimetric method was employed which necessitated the inclusion of 200 mg of sodium citrate per 10 ml of the medium.

Thymol blue was the indicator used. The *L. casei* factor was measured by the method of Teply and Elvehjem ('45).

Samples were prepared for both assays in the same way by incubation with takadiastase (0.1 gm of enzyme per 5 gm of sample) at 37°C. for 24 hours under toluene. The digests were then neutralized and autoclaved at 15 points pressure for 15 minutes (Luckey, Briggs, Moore, Hart and Elvehjem, '45; and University of Texas Publication no. 4237, '42).

#### RESULTS AND DISCUSSION

The results of this survey are summarized in table 1. The values reported are in terms of micrograms of the vitamin per 100 gm of the entire, original contents of the can except in the case of salmon where the brine was discarded.

The pyridoxine values were considerably higher than those for biotin and folic acid. The range for pyridoxine in individual samples of any product was three-fold or less in all instances with the exception of grapefruit juice and salmon where it was four-fold. Salmon contained two or three times as much pyridoxine as the vegetables and almost ten times as much as the fruit.

Fruit contained the smallest amount of biotin while the fish product contained the largest. The range of this factor was observed to be rather small, being three-fold or less in all of the foods analyzed except peaches.

The *S. lactis* factor of the folic acid complex showed the same range of values as biotin while the values for *S. lactis* factor averaged 30 to 50% of the *L. casei* factor content. However, both of them were present in much greater amounts in spinach and green asparagus, and in lower amounts in salmon than was biotin. In green asparagus, yellow corn, grapefruit juice, peas and salmon the range was two-fold or less for the *L. casei* factor while this small range occurred only in green asparagus and yellow corn samples for the *S. lactis* factor. The greatest range for the *L. casei* factor was four-fold in spinach while the greatest ranges for the *S. lactis* factor were seven-fold in green beans and four-fold in salmon and spinach.

TABLE 1  
*Pyridoxine, biotin, S. lactis factor and L. casei factor content of canned foods on the fresh weight basis.*

PRODUCT	NO. OF SAMPLES	PYRIDOXINE		BIOTIN		S. LACTIS		L. CASEI	
		Range	Ave.	Range	Ave.	Range	Ave.	Range	Ave.
		$\mu\text{g}/100\text{ gm}$		$\mu\text{g}/100\text{ gm}$		$\mu\text{g}/100\text{ gm}$		$\mu\text{g}/100\text{ gm}$	
Asparagus, green	10	17-48	30	1.0-2.1	1.7	3.2-7.9	5.8	6.2-11.2	9.0
Beans, green	11	18-52	32	1.1-1.8	1.3	1.2-8.3	2.9	4.0-11.0	7.7
Carrots	10	14-30	22	1.0-2.0	1.5	1.0-2.2	1.3	2.2-5.7	4.1
Corn, yellow	10	41-100	68	1.4-2.7	2.2	1.3-2.4	1.7	3.3-6.9	5.6
Grapefruit juice	11	7-27	14	0.3-0.4	0.3	0.3-0.7	0.5	1.0-1.6	1.2
Peaches	9	11-20	16	0.05-0.3	0.2	0.3-1.2	0.5	1.0-2.9	1.5
Peas	10	24-72	46	1.4-3.5	2.1	1.0-2.2	1.7	3.2-5.2	4.4
Salmon	10	70-260	130	6.7-15.2	9.8	1.0-4.4	2.6	5.3-10.2	6.9
Spinach	10	45-80	60	1.1-3.1	2.3	4.0-16.5	7.4	11-51	20.7
Tomatoes	10	47-98	71	0.8-2.4	1.8	1.5-4.1	2.7	3.9-8.2	5.4

The ranges of the other products were three-fold or less for both factors.

The per cent distribution of these vitamins between the solids and the liquid of the can is shown in table 2. Pyridoxine distribution was approximately 60% in the solids and 34% in the liquid. This distribution is essentially the same as that found for thiamine and riboflavin by Brush et al. ('44). Seventy-seven to 99% of the biotin was found in the drained solids in all products analyzed except tomatoes. Due to the

TABLE 2

*The distribution of pyridoxine, biotin, S. lactis factor, and L. casei factor between the solids and liquids of canned foods.*

PRODUCT	WEIGHT	PYRIDOXINE	BIOTIN	S. LACTIS FACTOR	L. CASEI FACTOR
% of total present in the solids <sup>1</sup>					
Asparagus, green <sup>2</sup>	62	65	91	68	65
Beans, green	60	63	85	67	64
Carrots	67	66	77	74	72
Corn, yellow	69	72	88	77	73
Peaches	65	67	89	80	78
Peas	63	64	88	83	66
Spinach	62	64	99	67	63
Tomatoes	57	66	68	63	66

<sup>1</sup> Average values for 5 different samples of each product obtained by separate analysis of the liquids and drained solids. The difference between the figures given and 100 represents the percentage found to be present in the liquid in each instance.

<sup>2</sup> Six samples analyzed.

high moisture content and fragile structure of the canned tomatoes, the separation of the drained solids was not as distinct on the drained weight screen as was the case with the other products. Biotin in the bound state is the least water soluble of any of the B-complex. Seventy-two to 80% of both the S. lactis and L. casei factors occurred in the solids of carrots, yellow corn, and peaches while the distribution was about two-thirds in the solids and one-third in the liquids in the other products. In general, except for biotin, there was rather close agreement between the percentages of the total weight contributed by the solids in the can and the percentages of these vitamins contained in the solids.

## SUMMARY

Pyridoxine, biotin and "folic acid" values on 101 samples of canned foods are reported in this paper. The distribution of these factors between the solids and the liquids of the product is also reported. Salmon, yellow corn, and tomatoes are good sources of pyridoxine. Approximately two-thirds of this vitamin was found in the solids of the product. Salmon had the highest biotin content. Sixty-eight to 99% of the biotin was present in the solids of the products analyzed. "Folic acid" measured as *L. casei* and *S. lactis* activity against crystalline vitamin B<sub>12</sub> was found in the highest quantity in green vegetables where about 64% of the factors occurred in the solids in each instance.

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# NUTRITIONAL STATUS OF RATS ON MILK DIETS CONTAINING SUCCINYLSULFATHIAZOLE <sup>1</sup>

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Feeding experiments with mineralized cows' milk have shown that a good nutritional status can be maintained in rats by such a diet (Underhill, Orten, Mugrage and Lewis, '33). Although some evidence suggests that milk is a good source of all the B vitamins, there is proof to the contrary (Williams, Cheldelin and Mitchell, '42; Day, Wakim, Zimmerman and McClung, '44). Furthermore, available data show that cows' milk is generally a poor source of vitamin K and that at times it may be almost entirely devoid of activity (Sells, Walker and Owen, '41; Mueller and Wertz, '45). Therefore, it is possible that a milk diet supports an intestinal flora favorable for the production of adequate amounts of such needed factors. Accordingly, this investigation was planned to determine the nutritional adequacy of milk for rats deprived of vitamins which are known to be synthesized by the normal intestinal flora. An attempt was made to prevent vitamin synthesis by a combination of cecectomy and succinyl-sulfathiazole (SST) feeding, but because the animals developed a severe diarrhea, this was abandoned. Therefore, only SST was used to inhibit vitamin synthesis.

<sup>1</sup> Supported in part by a grant from the Research Fund of the Graduate School of Indiana University.

## EXPERIMENTAL

Weanling piebald rats from our stock colony were used and were divided as equally as possible on the basis of litter membership, sex and weight. Most of the rats were housed in individual cages with screen floors, but a few were kept in screen-bottom group cages with 3 or 4 animals per cage.

Evaporated milk<sup>2</sup> and powdered whole milk,<sup>3</sup> purchased on the open market, were used. To mineralize the powdered milk, 1.2 gm of ferric citrate, 200 mg of manganous chloride, and 10 mg of copper sulfate were mixed with 1000 gm of the milk powder. Rats given evaporated milk were supplemented daily except Sundays, with 0.2 mg of copper, 3.4 mg of manganese and 2.4 mg of ferrous iron, each as the sulfate, in concentrated aqueous solution. The solutions were pipetted into small amounts of milk in food cups. Later in the day milk was allowed ad libitum. Drinking water was available to all animals.

The insoluble sulfonamide was administered as the sodium salt in the experiments involving evaporated milk. This was prepared by suspending 40 gm of SST in 165 ml of water and then adding 15 gm of sodium bicarbonate. As soon as the SST was completely dissolved and carbon dioxide ceased to evolve, the clear solution was stored in a brown bottle. Each day the required amount of SST solution was added to milk from freshly opened cans and mixed with it by vigorous stirring. The milk for control animals given no SST was treated similarly by adding equivalent amounts of sodium bicarbonate in solution.

## RESULTS

The effects on growth of rats given SST in amounts ranging from 0 to 10% of the total milk solids fed are summarized in table 1. Growth impairment occurred at SST levels as low as 4%. The effect was noticeable from the second week

<sup>2</sup> Wilson's irradiated evaporated milk (Indianapolis, Indiana).

<sup>3</sup> The powdered whole milk was purchased from the Hoosier Condensed Milk Company, Bluffton, Indiana.

and became progressively more pronounced with time. After 6 to 9 weeks, at the higher levels of SST, growth became almost stationary in most of the animals. However, a small percentage increased in weight almost as rapidly as the controls which received no SST. A few animals died after 10 to 14 weeks. The few rats which developed severe urinary tract concretions suddenly refused food and died within a few days. In others which gradually declined and died, gross examination revealed no evidences of such concretions.

TABLE 1

*Effect of succinylsulfathiazole on the growth of rats fed mineralized milk.*

NO. OF RATS	SEX	LEVEL OF SST IN DIET <sup>1</sup>	AVERAGE WEEKLY WEIGHT						
			0	2	4	6	8	10	12
			gm	gm	gm	gm	gm	gm	gm
Evaporated milk									
16	M	10	48	78	103	123	129	138	140
21	M	5	50	89	122	146	174	176	167
9	M	0	40	80	131	185	225		
20	F	10	51	75	93	104	110	121	119
21	F	5	42	71	99	118	130	128	
8	F	0	36	76	108	137	155	175	185
Dried milk									
5	M	8	52	103	141	157	153	156	
9	M	4	52	110	175	224	253	263	277
9	M	0	49	117	185	251	282	315	326
4	F	8	47	89	113	120	124		

<sup>1</sup> Grams per 100 gm of milk solids.

Observations were made on the effects of "folic acid" and a liver concentrate on the growth of rats fed SST in dried or evaporated milk. "Folic acid" was prepared for administration, by dissolving the powder in water in such amounts that the solution contained 90  $\mu$ g per milliliter. In most cases 18  $\mu$ g were given per rat daily for a minimum of 3 weeks after the animals had been on the experimental diets 4 to 10 weeks. Larger amounts of "folic acid" seemed to have no greater effect on the growth or appearance of the rats. The

liver concentrate was prepared for administration as an aqueous solution containing 0.4 gm per milliliter. The dosage was 0.2 gm per rat daily.

The results summarized in table 2 clearly show a definite growth response in SST-fed rats given "folic acid" or liver. Animals given the larger amounts of SST showed a greater growth response upon administration of either supplement. No improvement in growth occurred in the SST-fed animals given a mixture of thiamine, riboflavin, pyridoxine, pantothenic acid, nicotinic acid and inositol. Likewise, feeding xanthopterin<sup>4</sup> did not improve the growth or appearance of the animals.

In some experiments the liver fraction was fed continuously for 10 to 12 weeks after restriction to the milk-SST diets. Under these conditions the growth and appearance of the animals was essentially the same as in controls not given SST. Furthermore, rats on the SST-free evaporated milk diets grew as rapidly as similar animals given liver concentrate regularly for the first 8 weeks. This suggests that liver concentrate furnishes nothing essential to the growth of milk-fed rats if synthesis of vitamins by intestinal microorganisms is unimpaired.

Prothrombin time determinations were made on diluted plasma (12.5%) from more than 100 rats given evaporated milk, and on 30 that were fed the dried milk. Changes from normal were found only in rats fed the SST at a level of 10%. Whereas none of the animals receiving less than 10% SST had prothrombin time values over 51 sec., the values were above 65 in a number of the 10% SST group, but many were in the normal range ( $40 \pm 2.2$  sec.) The plasma prothrombin time returned to the normal range following the administration of 2-methyl-1, 4-naphthoquinone.

Bacterial counts were made on the cecal contents of rats that had been on the different diets for 70 to 150 days. Appropriate dilutions were prepared in sterile tap water and

<sup>4</sup> The xanthopterin was synthesized by Drs. P. H. Hidy and F. W. Neumann, using the Koschura ('43) method.

TABLE 2

*Effect of supplements on the growth of rats fed mineralized milk plus succinylsulfathiazole.*

NO. OF RATS AND SEX	LEVEL OF SST IN DIET <sup>1</sup>	SUPPLEMENT ADDED	DAYS ON DIET BEFORE ADDING SUPPL.	AVERAGE GAIN BEFORE AND AFTER ADDING THE SUPPLEMENT					
				Days before <sup>2</sup>			Days after		
				21	14	7	7	14	21
				gm	gm	gm	gm	gm	gm
Evaporated milk									
10(5M)	10	“Folic acid”	56	10	2	— 3	11	26	43
9(4M)	10	None	54	16	9	5	3	4	7
4(1M)	10	Xanthopterin <sup>3</sup>	57	— 1	2	3	— 12	— 24	
3(0M)	10	None	57	0	7	1	— 7	— 16	
6(6M)	5	“Folic acid”	69	21	8	2	23	39	53
6(4M)	5	“Folic acid”	52	29	14	0	9	28	44
3(3M)	5	None	49	25	12	9	5	14	13
3(3M)	0	None	47	51	29	17	22	49	57
3(0M)	5	Vitamin mixt. <sup>4</sup>	74	0	7	2	5	6	
3(0M)	5	None	74	15	9	3	8	18	
2(2M)	0	“Folic acid”	35	45	26	8	23	55	82
2(2M)	0	None	35	63	28	22	33	58	99
5(1M)	0	Liver extract	52	32	19	3	13	24	41
5(1M)	0	None	52	41	20	15	17	28	42
Dried milk									
4(2M)	8	“Folic acid”	54	10	10	5	22	32	
9(4M)	8	Liver extract	31	51	21	11	14	30	43
8(4M)	8	None	32	53	22	8	4	1	7
5(4M)	4	Liver extract	62	30	16	7	4	18	29
6(5M)	4	None	62	37	19	8	3	9	17
5(4M)	0	Liver extract	41	60	39	22	8	20	30
7(5M)	0	None	33	66	48	27	19	26	36

<sup>1</sup> Grams per 100 gm of milk solids.

<sup>2</sup> This refers to gain occurring only during the indicated period of time prior to the beginning of supplements.

<sup>3</sup> 200 µg per rat daily.

<sup>4</sup> Thiamine hydrochloride, 100 µg; riboflavin, 200 µg; pyridoxine, 100 µg; calcium pantothenate, 200 µg; nicotinic acid, 200 µg; and inositol, 400 µg per rat daily.

duplicate plates were made using eosin methylene blue agar for the coliform group and a special medium for the estimation of the total population. The latter medium (pH 7.2 to 7.4) contained yeast extract, 5 gm; glucose 5 gm; peptone, 5 gm;  $K_2HPO_4$ , 1 gm; liver infusion, 200 ml; and distilled water, 800 ml.

Rats which were not fed SST had approximately 3 to 12 million coliform organisms per gram of cecal contents whereas those given 5 or 10% SST had less than 100,000. The exact coliform count was not determined in many instances because 1-50,000 was the lowest dilution plated; in these cases such plates showed no coliform organisms. The total count on the controls not fed SST averaged over 800,000,000 per gm of cecal material. The bacterial count on the animals receiving 10% SST averaged about 400,000,000, and it was approximately 600,000,000 for the 5% SST group. Thus the total bacterial count was affected less than the coliform count. Nevertheless, the total number of bacteria decreased as the amount of SST was increased.

#### DISCUSSION

The growth data obtained by us do not agree with those of Welch and Wright ('44), and Wright, Skeggs, Welch, Sprague and Mattis ('45), who noted little if any growth impairment in rats fed mineralized dried milk (Klim) and SST. The reason for the difference is not apparent.

The findings indicate that much more of the sulfonamide is necessary under these conditions to effect marked growth impairment than when used in purified diets to which no p-aminobenzoic acid is added. It appears that this might be due, in part at least, to the effect of p-aminobenzoic acid which is present in considerable amounts in milk (Landy and Dicken, '42). This substance reduces the bacteriostatic action of SST (Day et al., '43). Since normally, "folic acid" may be synthesized in the intestinal tract, the presence of p-aminobenzoic acid would be expected to promote "folic acid" synthesis. Thus a larger concentration of SST would be required in milk

to produce growth impairment comparable to that in animals on p-aminobenzoic acid-low diets.

The slight prolongation in prothrombin time may be regarded as due to the combined effects of reduced vitamin K synthesis in the intestinal tract and a low concentration of this vitamin in evaporated milk. However, there is some evidence that the change may also be related to an effect of SST on the liver enzyme (s) responsible for prothrombin formation. Pilgrim and Elvehjem ('45) showed that feeding of SST to rats on purified diets resulted in sufficient hepatic injury to markedly reduce the activity of succinoxidase, malic oxidase and cytochrome oxidase.

#### SUMMARY

Weanling rats were restricted to mineralized evaporated and mineralized dried whole milk diets to which succinylsulfathiazole (SST) was added in amounts up to 10%.

Growth was markedly impaired at a level of 10% SST. The effect was less severe at lower concentrations. Either "folic acid" or solubilized liver concentrate caused prompt growth resumption. Xanthopterin alone, and a mixture of thiamine, riboflavin, pyridoxine, nicotinic acid, calcium pantothenate, and inositol, had no effect.

A slight prolongation in the prothrombin time occurred in animals given 10% SST. This returned promptly to normal when 2-methyl-1, 4-naphthoquinone was fed.

Marked reduction in the concentration of coliform bacteria in the cecum occurred in animals given SST. The total bacterial count was appreciably decreased.

The results are interpreted as further evidence of a low concentration of "folic acid" and vitamin K in evaporated and dried cows' milk.

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# APPARENT PROLONGATION OF THE LIFE SPAN OF RATS BY INTERMITTENT FASTING<sup>1</sup>

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## ONE FIGURE

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## INTRODUCTION

When a sufficient amount of choice food is available, laboratory rats, like many humans, eat enough to become more or less obese. As a consequence, the life span of rats feeding ad libitum, like the life span of their human counterparts, is presumably shortened. This inference is supported by the repeated findings of McCay and his associates ('42 a, b; '43) that the life span of rats can be considerably prolonged by a drastic restriction in their allowance of food. The findings of McCay and his associates practically constitute an experimental confirmation of the claims of Cornaro (Butler, '05) who attributed a considerable prolongation of his life to a rigid restriction of his food intake. However, since the time of Cornaro (1464-1566) no similarly prolonged and rigid voluntary restriction of the human food intake appears to have been recorded. Obviously, Cornaro's prolonged practice of food restriction has not been widely followed because a normal appetite tends to impel its more or less complete appeasement at reasonably frequent intervals, when sufficient palatable food is easily obtainable. Only short periods of food restriction, such as the religiously interdicted periods of food restriction or fasting of the past, would seem to be practical.

<sup>1</sup> This research was aided by a grant from Swift & Co., Chicago.

In the future however, a periodic practice of food restriction or fasting is likely to depend mainly on experimental evidence of its value. It therefore seemed of interest to determine whether periodic or intermittent fasting would serve to prolong the life span of rats or reduce or prevent the shortening of the life span which is presumably produced by feeding *ad libitum*.

A study was already made by Robertson, Marston and Walters ('34) of the effect of intermittent fasting on the life span of mice. In that study, twenty-four male and twenty-four female mice were fasted 2 successive days in 7. The average life span of the fasted males was found to be 745 days while that of twenty-four controls was 712 days. The average for the fasted females was 819 days while that of twenty-four control females was 773 days. However, the prolongation of life was not regarded as significant by Robertson and his associates. One criticism of their study is that littermate mice were not used as controls. Hence the individual life spans apparently varied too much to make the results seem significant. Another criticism is that no observations appear to have been made to determine whether the fasted mice remained free from peptic erosion or ulceration of the stomach and duodenum. This has been found to occur in some mice (and young rats) after single periods of starvation of 36 hours or more (Sun, '27; Hoelzel and Da Costa, '37).

The effect and after-effect of intermittent fasting on some aspects of growth and nutrition were also studied by von Seeland on chickens (1887), by Morgulis on salamanders ('13), by Kopec and Latyszewski on mice ('32) and by Kellermann on rats ('39) but the effect on the life span was not determined in any of these studies.

Observations previously made in this laboratory showed that rats fasted every other day and fed a diet low in protein between fasts developed peptic ulcers in the forestomach within about 2 weeks (Hoelzel and Da Costa, '32). However, rats fed a diet adequate in protein between single-day fasts usually remained free from peptic ulcers. With the use of a

diet relatively high in protein, no complication with peptic ulceration was therefore expected to develop in rats fasted 1 day in 3 or 4 but some doubts were still entertained whether rats could be fasted 1 day in 2 during prolonged periods without peptic lesions developing. In man, ulceration of the stomach is far less likely to occur while fasting because of an apparently lower fasting gastric acidity and the absence of the forestomach. Fasting 1 day in 2 or 3 by man also is apparently not the equivalent of fasting 1 day in 2 or 3 by the rat. However, in personal experiments, one of us (H.) found it impossible to maintain normal energy or remain free from nutritional edema while fasting every other day during periods of 2 to 5 months (Hoelzel, '43) but fasting 1 day in 3 immediately after having fasted 1 day in 2 during 5 months led to a recovery of energy and disappearance of nutritional edema. It was also found possible to recover fully from a 33-day fast in less than 33 days (Hoelzel, '44). Under these circumstances, it was deemed advisable to try various amounts of fasting in determining whether intermittent fasting would prolong the life span of rats.

In addition to various amounts of fasting, it also seemed advisable to try several diets. As a result, this study became somewhat complicated by the number of variables involved. The object of the present communication, however, is to report only the results of intermittent fasting on the life span of rats, independent of the specific effects of the different diets that were tried.

#### METHODS

In this study, 137 rats (60 males and 77 females), raised in the laboratory from rats obtained from The Wistar Institute, were used. These were all of the rats in seventeen litters with two or more of one sex or both sexes raised. The seventeen litters consisted of fourteen first litters and three second litters, with from two to thirteen raised rats in the individual litters. The rats were not weaned completely (separated from their mothers) until they were 35 days old.

Three omnivorous diets and one vegetarian diet were used. The omnivorous diets were a basic diet and two diets with 10% bulk-formers added to the basic diet. The basic diet consisted of 61.5% cooked and dried "whole veal," 31% corn starch, 2% powdered yeast, 1% cod liver oil, 1.5% inorganic salt mixture and 3% veal bonemeal. This diet provided approximately 35% proteins. The cooked and dried "whole veal" included practically all of the edible parts of calves, excepting excess fat and blood.<sup>2</sup> The first lot of this prepared veal contained 52% protein and 40% fat. Less fat was included in the preparation of subsequent lots but the composition of the original lot was approximated by adding fat when the diets were prepared. The second omnivorous diet consisted of the basic omnivorous diet plus 10% finely ground alfalfa stem meal. The third omnivorous diet consisted of the basic diet plus 5% psyllium seed husks and 5% specially prepared kapoc. The kapoc was mechanically cleaned, ground, boiled, washed, partly bleached, again washed and dried. The vegetarian diet consisted of 50% whole wheat flour, 10% peanut flour, 7% lima bean flour, 7% wheat gluten flour (containing 80% gluten), 7% corn gluten meal, 7% linseed meal, 5% powdered yeast, 5% alfalfa leaf meal and 2% NaCl. This diet provided approximately 30% proteins. Lettuce trimmings were supplied practically daily as a supplement to all of the diets. The control rats and the intermittently fasted rats while fed were kept continuously supplied with food.

Before the rats were 42 days old, all of them were supplied with the same food. This included some of each of the four experimental diets. When the rats became 42 days old, they were distributed so that some littermates of the same sex served in littermate tests of the effect of intermittent fasting or different amounts of intermittent fasting while other littermates served in tests of the effect of the different diets. Some rats with more than one littermate of the same sex consequently served as one of the littermates in 2 or more kinds of littermate tests. The intermittent fasting included fasting 1

<sup>2</sup> This was specially prepared for us by Swift and Co., Chicago.

day in 4, 1 day in 3 and 1 day in 2. The fasting was begun at the age of 42 days and was continued until the rats died.

## RESULTS AND DISCUSSION

### *Effect of intermittent fasting on the life span*

Figure 1 shows the distribution of the life spans of the individual control and fasted rats in 25-day periods. The life spans of rats with littermates only in the same group (but on different diets) are included. The average life spans of the

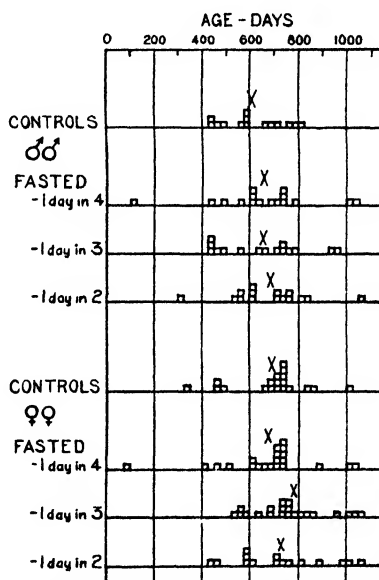


Fig. 1 Distribution of individual life spans of control and fasted rats in 25-day periods. Each square represents one rat. The average life span of each group is indicated by X. The life spans of rats with littermates only in the same group (but on different diets) are included.

groups are also indicated in figure 1 and in table 1. The results of the different amounts of fasting on littermates alone, with littermate controls fed identical diets ad libitum, are presented in table 2. Figure 1 and tables 1 and 2 show that, with the exception of the females fasted only 1 day in 4, the average life spans of all of the groups of fasted rats exceeded

that of the controls. Moreover, the data in table 2 indicate that the prolongation of life by fasting was practically proportional to the amount of fasting and that the life spans of the males were, on the average, increased more than the life spans of the females. However, this may merely mean that the life spans of the males were shortened more than the life spans of the females by feeding ad libitum.

The effect of fasting on littermate rats is also indicated by comparisons between the life spans of rats fasted 1 day in 4 or 3 and littermates fasted 1 day in 3 or 2, respectively. Data thus obtained and combined with the data in table 2 yield a

TABLE 1

*Average life spans of control and fasted rats. These averages include the life spans of rats with littermates only in the same group but on different diets.*

*Figures in parentheses indicate number of rats in each group.*

SEX	AVERAGE LENGTH OF LIFE IN DAYS				
	Controls	Fasted			
		1 day in 4	1 day in 3	1 day in 2	All degrees
Males	612 (14)	658 (16)	653 (15)	683 (15)	664 (46)
Females	688 (19)	675 (21)	781 (22)	733 (15)	730 (58)

total of thirty male littermate comparisons and show that the average life span of the males was increased 90 days by fasting. Similarly obtained data on females show that the average life span in forty-five littermate comparisons was increased only 23 days by fasting. It seems noteworthy that, in spite of the substantial increase in the average life span of the males by fasting, the life span of the fasted males only approximated that of the control females (fig. 1 and tables 1 and 2).

A more detailed analysis of the results suggests that fasting 1 day in 4 and 1 day in 2 were complicated more by some extraneous factors than fasting 1 day in 3 or feeding ad libi-

tum. Thus, figure 1 shows that the earliest male and female deaths occurred in the groups fasted 1 day in 4 and the impression was that some of the other rats fasted 1 day in 4 also did not fare as well as most of the rats fasted 1 day in 3 or the controls. Perhaps the amount of food consumed in 3 days of feeding, with increased voracity but without proportionately increased capacity after 1 day of fasting, constituted a greater physiological overstrain than the amount of food consumed by the controls or by the rats fasted 1 day in 3. Figure 1 further shows that the males and females fasted 1 day in 2 also began dying earlier than the rats fasted 1 day in 3. Evidently fasting 1 day in 2 and beginning this at the age of 42 days was too much fasting for some rats. One of the females fasted 1 day in 2 apparently died of a hemorrhage from a chronic duodenal ulcer. Nothing like this was seen among over 2000 rats in a study of the production of peptic ulcers (Hoelzel and Da Costa, '37). More or less erosion and ulceration of the stomach was observed in other rats in this study but in most cases the lesions merely seemed to be due to premortal conditions, chiefly starvation due to loss of appetite associated with, or produced by, respiratory infections. (In about half of the rats with respiratory infections, no erosion or ulceration of the stomach occurs in spite of a complete loss of appetite or starvation and the usual postmortem changes in the intestines also are not seen. In such cases, all digestive secretions seem to be suppressed, excepting the secretion of a little bile.) Some erosion of the stomach may have occurred as a direct result of the experimental fasting among the rats fasting 1 day in 2 while they were still young. Moreover, female rats were previously found to develop more severe gastric lesions than males during prolonged starvation and a complication of this type may therefore explain why the females in this study did not benefit as much as the males from repeated single-day fasts. However, individual rats vary in their susceptibility to peptic erosion and ulceration and even some that were fasted 1 day in 2 evidently remained entirely free from such lesions. Both the male and the female



that lived longest among the 137 rats (1057 and 1073 days, respectively) were rats fasted 1 day in 2. The optimum amount of fasting for the average rat in this study nevertheless appears to have been fasting 1 day in 3 and the data in table 2 show that with this amount of fasting the life span of the males was increased about 20% and that of the females about 15%.

### *Effect of intermittent fasting on growth*

Table 2 shows that the average weights of the intermittently fasted rats at 300 days were always lower than the average weights of their littermate controls but no drastic retardation of growth was produced by the fasting. In some cases, the average femoral lengths of the fasted rats at death were greater than, or equal to, those of the controls and, in other cases, the rats were only a little smaller. In short, intermittent fasting seems to make it possible to increase the life span to some extent without stunting the rats. Tests are in progress

TABLE 2

*Showing the effect of different amounts of fasting on the weight, size (length of femur at death) and life span of littermate rats. Littermate controls fed identical diets ad libitum.*

AMOUNT OF FASTING	NUMBER OF PAIRS OF LITTER MATES	AVERAGE WEIGHT (GM)				AVERAGE FEMORAL LENGTH AT DEATH (MM)		AVERAGE LENGTH OF LIFE (DAYS)		
		At 42 days		At 300 days						
		Controls	Fasted	Controls	Fasted	Controls	Fasted	Controls	Fasted	Difference due to fasting
Males										
1 day in 4	7	149	149	449	413	39.1	39.3	681	768	+ 87
1 day in 3	7	133	133	397	339	38.7	36.7	557	667	+ 110
1 day in 2	4	118	127	356	265	38.1	36.0	527	666	+ 139
All degrees	18	136	138	408	357	38.7	38.1	599	706	+ 107
Females										
1 day in 4	12	117	123	280	248	34.6	35.1	721	685	- 36
1 day in 3	10	118	120	291	258	34.8	35.2	708	814	+ 106
1 day in 2	5	113	114	276	234	34.5	34.1	614	768	+ 154
All degrees	27	117	120	283	249	34.7	35.0	696	748	+ 52

to determine whether the size of fasted rats can be more fully maintained and life can still be prolonged by beginning the fasting at 100 or 200 days, instead of at 42 days, and fasting the rats no more than 1 day in 3.

*Are the results of fasting due, in whole or in part,  
to increased activity?*

Tests made with rotary cages by Wald and Jackson ('44) revealed that rats run more when deprived of food, and McCay and his associates ('41) found that exercised rats lived longer than non-exercised rats. The activity of our fasting rats was largely limited to gnawing at the cages in attempts to escape. Many of the rats that were fasted 1 day in 4 or 3 seemed to become adapted to the fasting and remained at rest most of the time. The greatest unrest was manifested by some of the rats that were fasted 1 day in 2 but the life spans of the most restless rats were not the longest. The explanation seems to be that the greatest unrest or gnawing was manifested by the most voracious rats — the rats that apparently ate the greatest amounts of food between the days of fasting. The amount of food eaten may therefore have more than offset any possible benefit from increased exercise. In any event, the finding of McCay and his associates that rats subjected to forced exercise lived longer than non-exercised rats did not prove that the exercise per se increased the life span. The periods of forced exercise may merely have served to prevent the rats from eating as much as the controls in relation to their respective physiologic needs.

*Influence of individual constitutions on the life spans*

Constitutional similarities and differences among the individual rats, as determined by genetic factors and pre-experimental nutritional conditions, were obviously important factors determining the specific life spans. In the first place, a high degree of genetic uniformity in the Wistar strain evidently explains the death of 67% of the rats between the

ages of 550 and 850 days and the death of 85% between the ages of 400 and 900 days in spite of the use of four different diets and four different regimens of feeding or fasting. Some littermate rats, after having been kept from 400 to 1000 days on widely differing nutritional regimens, died within 24 hours or a few days of one another. Four of the twelve rats that lived to be over 1000 days old belonged to one of the seventeen litters. The view that rats in small litters are likely to be in a superior condition is supported by the finding that two males that composed one of the two smallest litters not only lived longer than eight (all) other males on similar nutritional regimens but also lived longer than fourteen (all) females on similar nutritional regimens. In contrast to this, the average life span of thirteen rats that composed one of the three largest litters was the lowest of any of the seventeen litters. However, independent of the size of the litters from which the rats came, the life span was found to be influenced more or less by the pre-experimental nutritional status or weight attained by the age of 42 days, when the rats were started on the specific experimental regimens. That is, the rats that were heaviest at the age of 42 days tended to live longest among the rats on the same regimen but this again was less true of the rats that were fasted 1 day in 3 or 2 than of the control rats or those that were fasted only 1 day in 4. That is, the prolongation of life due to fasting 1 day in 3 or 2 tended to outweigh the apparent handicap of a poorer nutritional start in life, as indicated by a lower than average pre-experimental weight. The data in table 2 show clearly that the heaviest group of male control rats lived longer than the lighter male controls and that their fasted littermates had correspondingly long life spans.

*Influence of intermittent fasting on the development  
of disorders leading to death*

Saxton ('45) showed that the development of inflammatory, neoplastic and degenerative diseases was delayed by the restriction of calories which increased the life span of the rats

in the experiments of McCay and his associates. Similarly, intermittent fasting seems to delay the development of the disorders which lead to death. Table 3 shows that a retardation of the development of mammary tumors, proportional to the amount of fasting, occurred in this study. These results support the observations previously made by Tannenbaum

TABLE 3

*Development of mammary tumors in control and fasted rats.*

	CONTROLS	FASTED		
		1 day in 4	1 day in 3	1 day in 2
Number of females developing tumors	7	6	8	1
Per cent of females developing tumors	37%	29%	36%	7%
Earliest age at which a tumor began developing	437 days	458 days	675 days	775 days
Average age at which tumors began developing	628 days	613 days	783 days	.
Average life span of females with tumors	760 days	760 days	871 days	977 days
Weight of largest tumor	462 gm	220 gm	140 gm	26 gm
Average weight of tumors	193 gm <sup>1</sup>	67 gm <sup>1</sup>	36 gm	..
Average rate of growth of tumors (gm gained per 100 days)	134 gm <sup>1</sup>	48 gm <sup>1</sup>	42 gm	13 gm

<sup>1</sup> The weights of the tumors in 2 control rats and in 1 rat fasted 1 day in 4 were not recorded and the weight of a tumor in another rat fasted 1 day in 4 was excluded because the tumor was dehydrated. It became dehydrated because it was torn loose by the rat after it apparently began to interfere with defecation.

('40; '42) concerning the relation between the food intake and the development of tumors in mice. However, genetic factors may explain the occurrence of mammary tumors in all of the (six) females in one of the seventeen litters and the occurrence of ten of the other sixteen tumor cases in five pairs of littermates. The larger tumors became responsible for the

deaths of some of the females because of the tendency of the large tumors to ulcerate or become obstructive. The development of other types of tumors was apparently also retarded by intermittent fasting. The only tumor in the digestive tract, a fibrosarcoma in the forestomach, was found in the oldest control rat (a female 1012 days old). The tumor in the stomach was probably secondary to a tumorous condition of the uterus. Ulceration of the tumorous uterus evidently was responsible for the development of a fistula between the uterus and the ileum in this rat. Nothing comparable to this was seen among the fasted rats.

#### SUMMARY

Tests in which a group of thirty-three rats were allowed the same food ad libitum and groups of thirty-seven, thirty-seven and thirty rats were fasted 1 day in 4, 3 and 2, respectively, after the age of 42 days, showed that the apparent life span was increased by the intermittent fasting. The optimum amount of fasting appeared to be fasting 1 day in 3 and this increased the life span of littermate males about 20% and littermate females about 15%. However, the pre-experimental condition of the individual rats was also found to be an important factor determining the life spans.

No drastic retardation of growth was produced by the intermittent fasting but the development of mammary tumors was retarded in proportion to the amount of fasting.

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# THE EFFECT OF DIFFERENT GRADES OF COCOA UPON THE RETENTION OF DIETARY CALCIUM BY GROWING RATS<sup>1</sup>

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Cocoa and chocolate are used extensively as flavoring materials in milk and ice cream, evidently in response to a consumer demand. Their use may be presumed to increase the consumption of these excellent calcium foods. The presence of 0.5 to 0.6% of oxalic acid in cocoa, however, suggests that cocoa, or chocolate, may impair the utilization of the calcium in milk and in ice cream by the formation of the very insoluble calcium oxalate, a form of calcium that is practically unavailable to the growing rat at least (Fairbanks and Mitchell, '38).

This possibility induced Mueller and Cooney ('43) to compare the retention of calcium by growing rats subsisting upon diets based upon whole milk powder with and without an addition of cocoa in the proportion of 0.84 part of the basal diet to 0.16 part of cocoa. The basal diet contained 0.554% of calcium and 0.472% of phosphorus. Twenty-one pairs of rats were used in addition to twenty-one litter-mate control rats killed and analyzed at the beginning of the experiment to permit an estimate of the initial content of calcium in the test rats. Within each pair of rats the consumption of the basal diet was equalized, while one of the rats received the cocoa powder in addition. After 5 weeks of feeding, the rats were sacrificed and analyzed for calcium and phosphorus.

<sup>1</sup> This investigation was aided by funds granted to the University of Illinois by the National Dairy Council on behalf of members of the chocolate industry.



The results of this well-conceived and well-executed experiment were clear-cut in their significance as revealed by statistical analysis. The rats fed the cocoa supplement, although receiving 19% more dry food, 6% more calcium and 30% more phosphorus than the rats on the basal diet only, grew only 89% as fast and deposited in their bodies only 76% as much calcium and 80% as much phosphorus. The effect of the cocoa supplement upon growth had been previously shown by Mueller ('42) to be traceable probably to its content of tannic substances. The primary effect upon mineral utilization was probably upon calcium, since (1) calcium was presumably a limiting factor in the diet, (2) calcium limitation will restrict phosphorus utilization, because the two minerals are so largely utilized together as a calcium phosphate in bone formation, and (3) the depression of phosphorus retention by cocoa was so nearly the same as that of calcium retention. However, the oxalic acid content of the cocoa could account for only about one-third of the observed depression in calcium retention. No clue to the residual causes of this depression was afforded by the data of the experiment or by the discussion of them by the authors. It should be noted that the ratio of milk solids to cocoa in the diets of this experiment was about 3.3 to 1, while in chocolate milk it is ordinarily about 13 to 1.

The cocoa that Mueller and Cooney used was a low-cost American process cocoa<sup>2</sup> containing 11.23% fat and 5.25% fiber. Cocos of this general description are common at times on the market, being used in the manufacture of bakery goods and for low-cost chocolate syrups and confections, though there seems to be a considerable sale of such products for domestic use by certain retail stores throughout the country.

#### PLAN OF EXPERIMENTS

These experiments of Mueller and Cooney were repeated on growing rats at the University of Illinois, using essentially the same technic, but studying three different kinds of cocoa,

<sup>2</sup> Personal communication.

i.e., the low-cost brand used by the Massachusetts investigators and two medium-cost products, an American process breakfast cocoa and a Dutch process cocoa. The composition of these cocoas, in so far as they were analyzed in this laboratory, are summarized in table 1. In the Illinois experiments, the cocoas were incorporated into the basal diets at the expense of sugar, instead of being added to the diets, and all diets were equalized in fat content. The composition of the diets is given in table 2.

The effects of the different cocoas were studied in two experiments. In the first experiment, the effect of the two

TABLE 1  
*The chemical composition of the experimental cocoas.*

DESCRIPTION OF COCOA	MOISTURE	ETHER EXTRACT	CRUDE PROTEIN	CRUDE FIBER	TANNIC SUBSTANCES <sup>1</sup>	THEOBROMINE	OXALIC ACID	ASH	CALCIUM	PHOSPHORUS
American process:	%	%	%	%	%	%	%	%	%	%
No. 1	4.70	22.45	22.62	3.90	10.57	1.79	0.504	4.97	0.121	0.668
No. 2 <sup>2</sup>	5.73	10.58	24.94	5.98	11.80	2.10	0.552	5.82	0.156	0.710
Dutch process:	5.20	23.33	21.12	3.61	6.62	1.44	0.512	8.50	0.124	0.640

<sup>1</sup> Corrected for ash.

<sup>2</sup> The same brand of cocoa used by Mueller and Cooney ('38).

medium-cost cocoas on the retention of the calcium and phosphorus of milk was studied with nine trios of rats, trio mates receiving equal amounts of their respective diets. In the second experiment, the effect of the low-cost cocoa was assessed with twelve pairs of rats, also receiving equal amounts of their respective diets. However, in both experiments the body weight gains of trio mates, or pair mates, were equalized by additions of sugar to the diet of the laggard rats, which were generally the rats receiving cocoa. This procedure was adopted because it could not be assumed that the net energy value of the cocoa was the same as that of the sugar it displaced in the cocoa diets. The experiments on individual pairs or trios were terminated after 4 to 9 weeks of feeding, de-

TABLE 2  
*The experimental diets.*

CONSTITUENTS:	EXPERIMENT 1			EXPERIMENT 2	
	Diet 617	Diet 618	Diet 619	Diet 637	Diet 638
	%	%	%	%	%
Defatted dried beef	30	30	30	25	25
Non-fat dry milk solids (spray process)	19.47	19.47	19.47	19.47	19.47
Cocoa, American process no. 1	0	16	0	0	0
Cocoa, Dutch process	0	0	16	0	0
Cocoa, American process no. 2	0	0	0	0	16
Salt mixture Ca- and P-free	2	2	2	2	2
NaCl	1	1	1	1	1
BaSO <sub>4</sub>	2	1.38	1.41	0	0
Wood Flock	0	0	0	2	1.04
Starch, vitaminized	5	5	5	5	5
Sucrose	28.62	16.83	16.94	16.80	10.12
Cerelose	0	0	0	16.80	10.13
Cod liver oil, fortified	1.5	1.5	1.5	1.5	1.5
Wheat germ oil	0.5	0.5	0.5	0.5	0.5
Lard	9.91	6.32	6.18	5	5
Mazola	0	0	0	4.93	3.24
Total	100.00	100.00	100.00	100.00	100.00
Calcium %					
Mix 1	0.225	0.244	0.242	0.268	0.292
Mix 2	0.261	0.275	0.277	0.272	0.292
Phosphorus %					
Mix 1	0.410	0.518	0.511	0.422	0.463
Mix 2	0.425	0.525	0.506		
Protein %					
Mix 1	31.62	25.06	35.00	27.19	30.75
Mix 2	31.50	35.12	34.88		
Calories per gm					
Mix 1	4.64	4.71	4.70	4.66	4.75
Mix 2	4.61	4.73	4.72		

pending upon the rate of growth in each trio or pair, and the carcasses were analyzed for calcium and, in the first experiment, for phosphorus also.

The calcium and phosphorus in diets, cocoas and carcasses were determined by slight modifications of the methods of the Association of Official Agricultural Chemists. In the cocoa analysis, the tannic substances were determined by the method of Mueller and Kuzmeski ('44), the theobromine by the method of Wadsworth ('21), and the oxalic acid by a slight modification of the method of Majumdar and De ('38).

#### THE EXPERIMENTAL RESULTS

The average growth data in the two experiments are summarized in table 3. The differences in final weight and gain among trio or pair mates were largely due to differences in intestinal fill, since final weights relate to the empty carcasses. The differences in average attained body length among rats on comparable diets were insignificant statistically in the first ex-

TABLE 3  
*Average growth of rats on the experimental diets.*

	EXPERIMENT 1			EXPERIMENT 2	
	Diet 617 No cocoa	Diet 618 American process cocoa no. 1	Diet 619 Dutch process cocoa	Diet 637 No cocoa	Diet 638 American process cocoa no. 2
Number of rats	9	9	9	12	12
Body weight —					
Initial, gm	47.0	46.0	47.2	47.2	47.4
Gain, gm	131.3	125.2	126.1	157.8	155.8
Body length, mm	198.7	199.6	199.9	206.1	203.6
Days on food	36	36	36	40	40
Total food eaten —					
Diet, gm	326.6	326.6	326.6	371	371
Sugar supplement —					
gm	3.8	24.4	20.1	0	70
pct. of diet	1.2	7.5	6.2	0	18.9

periment, but in the second experiment, the attained body length of the control rats receiving no cocoa averaged 2.5 mm greater than that of the rats receiving the low-cost cocoa, and this difference was significant with  $P = 0.010$ .

The amount of extra sugar required to maintain gains in the cocoa rats equal to those in the non-cocoa rats, in per cent of the basal food consumed, averaged 18.9 for the low-cost American process cocoa no. 2, 6.3 for the American process breakfast cocoa no. 1, and 5.0 for the Dutch process cocoa. The

TABLE 4  
*The average calcium metabolism data.*

	EXPERIMENT 1			EXPERIMENT 2	
	Diet 617 No cocoa	Diet 618 American process cocoa no. 1	Diet 619 Dutch process cocoa	Diet 637 No cocoa	Diet 638 American process cocoa no. 2
Number of rats	9	9	9	12	12
Total calcium consumed, gm	0.763	0.821	0.817	1.002	1.084
Final calcium content, gm	1.203	1.130	1.111	1.453	1.273
Initial calcium content, gm	0.502	0.491	0.504	0.596	0.599
Calcium retained, gm	0.701	0.639	0.606	0.858	0.875

difference in this respect between the low-cost cocoa and the two medium-cost cocoas is highly significant, while the percentages for the two medium-cost cocoas were indistinguishable statistically.

The average calcium metabolism data are presented in table 4. Although receiving more dietary calcium than their litter-mate controls on cocoa-free diets, the cocoa rats stored considerably less calcium in their bodies in all trios and pairs. This situation is analyzed in detail for the individual rats in table 5, with statistical evaluations of average group differ-

TABLE 5  
*The effect of cocoa on the utilization of dietary calcium by growing rats.*

PAIR OR TWO NO.	AMERICAN PROCESS COCOA NO. 1						DUTCH PROCESS COCOA						AMERICAN PROCESS COCOA NO. 2					
	No-cocoa rats			Cocoa rats			No-cocoa rats			Cocoa rats			No-cocoa rats			Cocoa rats		
	Calcium retention in per cent of intake			Calcium retention in per cent of intake			Calcium retention in per cent of intake			Calcium retention in per cent of intake			Calcium retention in per cent of intake			Calcium retention in per cent of intake		
	Total intake	Intake of cocoa	Intake of non-oxalate calcium	Total intake	Intake of cocoa	Intake of non-oxalate calcium	Total intake	Intake of cocoa	Intake of non-oxalate calcium	Total intake	Intake of cocoa	Intake of non-oxalate calcium	Total intake	Intake of cocoa	Intake of non-oxalate calcium	Total intake	Intake of cocoa	Intake of non-oxalate calcium
1	95.2	74.6	80.5	86.3	95.2	77.6	83.3	88.6	88.3	68.3	73.9	77.5	88.3	68.3	73.9	77.5	88.3	68.3
2	99.3	88.9	95.5	101.9	99.3	89.3	95.6	100.0	91.8	68.8	74.4	78.1	91.8	68.8	74.4	78.1	91.8	68.8
3	94.6	74.6	80.3	85.8	94.6	68.5	73.4	78.1	88.2	61.4	66.4	69.6	88.2	61.4	66.4	69.6	88.2	61.4
4	96.9	78.5	84.3	89.9	96.9	61.7	66.0	70.1	79.3	68.1	73.7	77.3	79.3	68.1	73.7	77.3	79.3	68.1
5	84.8	84.8	91.8	98.7	84.8	85.0	91.3	97.4	92.5	64.1	69.3	72.7	92.5	64.1	69.3	72.7	92.5	64.1
6	72.9	67.1	71.9	76.6	72.9	73.6	78.6	83.4	85.6	65.1	70.9	74.8	85.6	65.1	70.9	74.8	85.6	65.1
7	95.0	76.2	81.7	87.1	95.0	61.7	66.1	70.2	79.2	55.5	60.0	63.0	79.2	55.5	60.0	63.0	79.2	55.5
8	100.0	77.1	83.0	88.9	100.0	76.0	81.4	86.6	84.1	64.2	69.4	72.8	84.1	64.2	69.4	72.8	84.1	64.2
9	88.1	83.2	90.1	96.9	88.1	81.8	87.9	93.7	81.5	57.3	61.9	65.0	81.5	57.3	61.9	65.0	81.5	57.3
10	..	..	..	..	..	..	..	..	87.9	54.3	58.8	61.7	87.9	54.3	58.8	61.7	87.9	54.3
11	..	..	..	..	..	..	..	..	85.8	57.5	62.2	65.3	85.8	57.5	62.2	65.3	85.8	57.5
12	..	..	..	..	..	..	..	..	85.8	59.8	64.7	67.9	85.8	59.8	64.7	67.9	85.8	59.8
Ave.	91.9	73.3	84.3	90.2	91.9	75.0	80.4	85.3	85.8	62.0	67.1	70.5	85.8	62.0	67.1	70.5	85.8	62.0
Mean deviation from control	13.6	8.35	2.43	4.58	0.0009	0.018	0.31	1.7	9.09	51	0.0041	0.026	0.12	0.0001	0.0001	0.0001	0.0001	0.0001
S	8	8.35	2.43	4.58	0.0009	0.018	0.31	1.7	9.09	51	0.0041	0.026	0.12	0.0001	0.0001	0.0001	0.0001	0.0001
t	8	8.35	2.43	4.58	0.0009	0.018	0.31	1.7	9.09	51	0.0041	0.026	0.12	0.0001	0.0001	0.0001	0.0001	0.0001
P	8	8.35	2.43	4.58	0.0009	0.018	0.31	1.7	9.09	51	0.0041	0.026	0.12	0.0001	0.0001	0.0001	0.0001	0.0001

ences. In this table, the calcium retentions for all rats are computed as percentages of the total calcium intakes, and for the cocoa rats, in percentages also, first, of the non-cocoa calcium in the diet, and, second, of the non-oxalate calcium, assuming that the oxalic acid of the cocoa combined with dietary calcium to its full capacity.

The average deviations of the percentage retentions of total calcium for the cocoa rats as compared with their controls were 23.8 for the rats receiving the low-cost American process cocoa no. 1, 13.6 for the rats receiving the medium-cost American process cocoa no. 2, and 16.9 for the medium-cost Dutch process cocoa, representing average depressions in total calcium retention of 27.7, 14.8 and 18.4%, respectively. Again, the detrimental effect of the low-cost cocoa exceeded statistically the effect of the two medium-cost cocoas. Each of the cocoas depressed significantly the utilization of the calcium in the basal diets, since the calcium retentions of the cocoa rats computed as percentages of the non-cocoa dietary calcium were significantly smaller than the percentage calcium retentions of trio-mate or pair-mate controls, but again the effect of the low-cost cocoa exceeded statistically those of the medium-cost cocoas.

When the calcium retentions of the cocoa rats are computed as percentages of the non-oxalate calcium in the diet, the values for the medium-cost cocoas still average less than the values for their controls, but the differences are insignificant, with probabilities that a random combination of uncontrolled factors could have produced as great or greater average differences than those observed, of 0.31 and 0.12. These probabilities are too large to be neglected and hence the differences may well have resulted from random factors only. It may be concluded, therefore, that the oxalic acid in these cocoas could have accounted for the total depression in calcium utilization brought about by their consumption. From the analyses of the cocoas given in table 1, it may be computed that these medium-cost cocoas contain enough calcium to combine with 54% of their contents of oxalic acid.

The low-cost American process cocoa no. 2 presented a different picture, however. In this case, the average retention of dietary calcium computed on the intake of calcium not bound by oxalic acid, is 70.5, as compared with a percentage utilization of 85.8 for the calcium in the non-cocoa diet, representing a difference that is highly significant with  $P=0.0001$ . The conclusion is clear, therefore, that with this cocoa the detrimental effect on calcium utilization is not due entirely to its content of oxalic acid, the effect of which is represented by the difference between 23.8, the depression in the percentage retention of total calcium, and 15.3, the depression in the percentage retention of non-oxalate calcium. Since this difference, 8.5, is about one-third of the total depression of 23.8, it appears that the oxalic acid in this cocoa accounts for only about one-third of its total depressing effect on calcium utilization. Thus, these results confirm completely those of Mueller and Cooney obtained with the same brand of cocoa.

The carcasses of the rats in the first experiment were analyzed for phosphorus as well as for calcium. Since the experimental diets contained about twice as much phosphorus as calcium (table 2), they were evidently not deficient in phosphorus. Nevertheless, the retention of phosphorus was definitely smaller in the rats receiving cocoa than in the control rats without cocoa. This depression in phosphorus was evidently occasioned by the smaller retention of calcium by the cocoa rats, since the two elements are to a large extent combined as a calcium phosphate in bone growth. For this reason, the ratios of retained calcium to retained phosphorus, with their standard errors, are nearly the same:  $1.370 \pm 0.037$  for the non-cocoa rats,  $1.314 \pm 0.032$  for the rats receiving the American process cocoa, and  $1.303 \pm 0.061$  for the rats receiving the Dutch process cocoa. Using a "Student" ('25) analysis on paired differences, the probability that a random combination of uncontrolled experimental factors would have brought about average differences in ratio as large or larger than those observed is 0.08 for the comparison of control rats and the rats receiving American process cocoa, and 0.11 for



the comparison of control rats and the rats receiving Dutch process cocoa. These probabilities are too large to neglect, but small enough to suggest that these cocoas depressed calcium assimilation somewhat more than they depressed phosphorus assimilation. The small amount of phytin reported in cocoa (Arbenz, '22) does not seem to have affected phosphorus utilization.

#### CONCLUSIONS

1. Two medium-cost breakfast cocoas containing 22 to 24% of fat, one an American process and the other a Dutch process cocoa, depressed calcium assimilation in growing rats in proportion to their contents of oxalic acid. The cocoas themselves contained enough calcium to combine with 54% of their content of oxalic acid.

2. A low-cost American process cocoa containing 10.58% of fat depressed calcium assimilation to an extent about three times as great as could be accounted for by its content of oxalic acid.

3. The latter cocoa depressed the growth of rats also to a distinctly greater extent than the former two cocoas.

4. No distinction between the two medium-cost cocoas with reference to their effects upon growth or calcium metabolism was established.

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# FAILURE OF FEATHER PIGMENTATION IN BRONZE POULTS DUE TO LYSINE DEFICIENCY

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## ONE FIGURE

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Turkey growers who raise bronze poults in complete confinement are frequently disturbed by the appearance of an unusual extent of white coloring in the primary and secondary wing feathers. The tail feathers may also be affected. The abnormal pigmentation is most apparent during the first 4 or 5 weeks, and gradually disappears as the birds become older, even though the diet and conditions of management remain the same (Carriek, '45).

Funk and Kempster ('40) observed a white feather development in bronze poults when 10% cottonseed meal or 10% corn gluten meal was used in the ration. Feather pigmentation was normal when soybean meal was similarly used in the ration. They also recorded that the abnormality diminished as maturity approached, and they postulated that the pigmentation failure was due to "a deficiency of some constituent which was required for rapid growth but was released for pigmentation when the growth rate diminished." These authors also suggested that "manganesed calcium" used in some of the rations may have been a causative factor. Roberts ('45) noted that white plumage developed in bronze poults when 20% soybean meal in the ration was replaced with ground corn.

A series of reports from the Oklahoma Experiment Station (summarized by Ewing, '43) indicated that low fiber rations caused a similar loss of pigment in bronze poults. However, it might be noted that the source of fiber used in these studies was alfalfa stem meal which may have contributed many factors in addition to fiber.

Excessive white feather formation has also been reported in growing chickens (McConachie, Graham, and Branion, '35; and Poley, '38) but in these reports it is not clear that the causative factor was the same as in the cases involving poults. Groody and Groody ('42) reported that pantothenic acid deficiency caused less feather pigmentation in chicks.

The object of this investigation was to study the possible causes of the loss of pigment from the feathers of poults, and to determine how this syndrome can be prevented.

#### EXPERIMENTAL

Standard bred bronze poults, hatched from the Borden Experimental Farm flock, were used in this study. The day-old poults were placed in electrically heated, wire floored, battery brooders. Throughout the test they received the designated diets and fresh tap water ad libitum. The first test was designed to study the effect of varying fiber and calcium content, and of the use of corn gluten meal upon the incidence of the white feather syndrome. The diets and a summary of the observations are shown in table 1. The feather pigmentation was judged on an arbitrary scale when 0 indicated normal color, 1 indicated slightly abnormal extent of white color on the feathers of a few poults in the group, 2 indicated all poults with slight degrees of pigmentation failure, 3 indicated moderate to severe loss of pigment, and 4 indicated very severe loss of pigment with extremely wide white bands on both wings and also on the tail feathers. Figure 1 shows the normal bronze pattern of the poults on diet 4213 and the abnormal white wing feathers of poults on diet 4215.

The data of Almquist ('45) and of Block and Bolling ('45) on the composition of soybean protein and of corn gluten

protein indicated large differences in the content of glycine and lysine. In the second test, crystalline glycine <sup>1</sup> and crude lactalbumin, respectively, were added to the corn gluten meal diet, no. 4215, in such quantities as to raise the intake

TABLE 1

*Observations on corn gluten meal, fiber content, and calcium content as possible causes of pigmentation failure in bronze turkey poult.*

INGREDIENTS	DIF 1					
	4213	4214	4215	4216	4217	4218
	%	%	%	%	%	%
Yellow corn meal	20.4	20.4	20.4	22.9	25.4	17.9
Pulverized oats	10.0	10.0	10.0	5.0	...	10.0
Rollod oats	.	.	.	5.0	10.0	..
Ground wheat	.	.	.	10.0	20.0	.
Standard middlings	10.0	10.0	10.0	5.0	.	10.0
Wheat bran	10.0	10.0	10.0	5.0	.	10.0
Alfalfa meal	7.5	7.5	7.5	3.75		7.5
Alfalfa leaf meal			.	1.25	2.5	
Meat and bone scrap	12.5	12.5	12.5	12.5	12.5	12.5
Sardine meal	2.5	2.5	2.5	2.5	2.5	2.5
Soybean meal	20.0	10.0	.	20.0	20.0	20.0
Corn gluten meal		10.0	20.0	.	.	..
Flaydyx <sup>1</sup>	2.0	2.0	2.0	2.0	2.0	2.0
Ladpro 100 D <sup>2</sup>	1.6	1.6	1.6	1.6	1.6	1.6
Ground limestone	2.5	2.5	2.5	2.5	2.5	5.0
Salt with I <sub>2</sub> and Mn	1.0	1.0	1.0	1.0	1.0	1.0
Calculated % Fiber	6.78	6.46	6.14	5.02	3.26	6.73
Calculated % Protein	24.8	25.0	25.2	24.5	24.2	24.6
Calculated % Lysine	1.23	1.02	0.81	1.18	1.13	1.22
No. poult.	35	35	35	35	34	34
Av. wt. of poult. at 6 weeks (gm)	466	589	605	665	631	464
Achroma score	0	1.5	3	0	0	0

<sup>1</sup> Poultry feed supplement containing whey solubles, used to supply B-complex vitamins.

<sup>2</sup> Poultry feed supplement containing fish solubles and fish liver and glandular meal, standardized to contain 100 A.O.A.C. units of vitamin D per gm.

of these amino acids to the levels which would be supplied if soybean meal had replaced the corn gluten meal. The preventive values of crystalline d-lysine monohydrochloride <sup>2</sup> and

<sup>1</sup> Dow.

<sup>2</sup> Merck.

of calcium pantothenate were also tested in conjunction with the high corn gluten meal diet. Two levels of ground limestone were also used in this series to determine any effect of varying the calcium content of the ration. Table 2 summarizes the diets and observations.



Fig. 1 The upper poult was raised on diet 4213 and shows the normal bronze pattern. The lower poult was raised on diet 4215 and shows the abnormal white wing feathers.

Table 3 summarizes a test in which the effectiveness of crystalline d-lysine (Block) was compared with soybean meal, yeast, and casein as food sources of lysine. Several modifications of the lysine deficient ration were produced by varying the protein sources used. The basal mixture had the following composition: Yellow corn meal 26.3, pulverized oats 10.0, standard middlings 10.0, wheat bran 10.0, dehydrated al-

falfa meal 5.0, Flaydry <sup>3</sup> 2.0, Ladpro 100 D <sup>4</sup> 1.6, ground limestone 2.5, and salt with added iodine and manganese 1.0. To this basal mixture were added the variables of interest indicated in table 3. Diet 4406 contained the least lysine of any diet used in this study.

TABLE 2

*Effect of certain supplements added to the corn gluten meal diet which causes feather pigmentation failure.*

PEN	SUPPLEMENT TO DIET 4215	CONTENT OF			NO. OF POULTS	AV. WT. AT AGE IN WKS.	ACHROMA SCORE
		Protein	Lysine	Ground Lime- stone			
		%	%	%		gm	
4269	Glycine, 1.25%	26.1	0.80	2.5	8	377 (5)	4
4270	Lactalbumin, 10.5%	27.3	1.18	2.5	8	396 (5)	0
4271	None	25.2	0.81	2.5	8	355 (5)	3.5
4354	None	25.2	0.81	2.5	6	182 (4)	4
4355	Calcium pantothenate 10 mg/lb	25.2	0.81	2.5	6	240 (4)	4
4356	Lysine, 1.8 gm/lb	25.6	1.20	2.5	6	340 (4)	0
4357	None	24.6	0.79	5.0	6	227 (4)	3
4358	Lysine, 1.8 gm/lb	25.0	1.19	5.0	6	296 (4)	0

The preventive value of fish solubles and of fish meal were investigated, and this test is summarized in table 4. It was considered desirable to study these materials because of their general acceptance and wide use in commercial poultry feeds. Almquist ('45) gives the lysine content of fish solubles as 4.9% of the crude protein, and of fish meal as 5.7% of the crude protein.

#### DISCUSSION

From the data presented in table 1, it seems obvious that the only cause of the white feather syndrome was the use of corn gluten meal. Among the differences in amino acid composition of soybean meal, which did not produce the syn-

<sup>3</sup> Poultry feed supplement containing whey solubles, used to supply B-complex vitamins.

<sup>4</sup> Poultry feed supplement containing fish solubles and fish liver and glandular meal, standardized to contain 100 A.O.A.C. units of vitamin D per gram.

drome, and of corn gluten meal, which did produce the syndrome, was that of the lysine content. Throughout the course of the tests outlined in this report, the use of protein sources which were good carriers of lysine prevented the pigmentation failure. Whenever proteins of low lysine content were substituted, the white feather syndrome was observed. The use

TABLE 3

*Effect of varying the source of protein on the incidence of the white feather syndrome.*

INGREDIENTS	D I E T								
	4398	4399	4400	4401	4402	4403	4404	4405	4406
	%	%	%	%	%	%	%	%	%
Basal mixture <sup>1</sup>	68.4	68.4	68.4	76.7	68.4	68.4	68.4	68.4	68.0
Meat and bone scrap	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	6.5
Sardine meal	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	..
Cottonseed meal	16.6	..	..	..	..	..	..	..	8.5
Soybean meal	..	16.6	..	..	..	..	..	..	..
Dried brewer's yeast	..	..	16.6	..	..	..	..	..	..
Casein <sup>2</sup>	..	..	..	8.3	..	..	..	..	..
Linseed meal	..	..	..	..	16.6	..	..	..	8.5
Corn gluten meal	..	..	..	..	..	16.6	16.6	16.6	8.5
Lysine, crystalline <sup>3</sup>	..	..	..	..	..	..	1.8 <sup>a</sup>	3.6 <sup>a</sup>	..
Protein %									
(calculated)	24.1	24.0	24.9	25.0	23.0	24.3	24.7	25.1	22.4
Lysine %									
(calculated)	0.92	1.14	1.20	1.24	0.88	0.79	1.18	1.58	0.69
No. of poults	12	12	12	12	12	12	13	13	13
Av. wt. at 5 weeks	317	404	328	357	265	227	394	348	253
Achroma score	1	0	0	0	0	1	1	0	3

<sup>1</sup> See text for composition of basal mixture.

<sup>2</sup> Difference made up with the yellow corn meal.

<sup>3</sup> Lysine additions are expressed as grams per pound. The lysine used was Block's d-lysine. 2 HCl.

of crystalline lysine was as effective in preventing the syndrome as were any natural sources of lysine. It therefore seems obvious that under the conditions of this study, lysine deficiency was the cause of the pigmentation failure. Of course, it must be recognized that not all cases of pigmentation failure are necessarily due to a lysine deficiency. This syndrome is only an indication and as such may have numerous causes.

TABLE 4  
*Study of fish solubles and fish meal and their effect upon incidence of the depigmentation.*

INGREDIENTS	DIET											
	4440	4441	4442	4443	4444	4445	4446	4472	4473	4474	4475	
Yellow corn meal	%	%	%	%	%	%	%	%	%	%	%	
Ground barley	20.40	18.40	16.40	..	..	..	..	..	..	..	..	
Pulverized oats	10.00	10.00	10.00	..	..	..	..	10.00	10.00	10.00	10.00	
Standard middlings	10.00	10.00	10.00	..	..	..	..	10.00	10.00	10.00	10.00	
Wheat bran	10.00	10.00	10.00	..	..	..	..	10.00	10.00	10.00	10.00	
Alfalfa meal	7.50	7.50	7.50	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	
Meat and bone scrap	12.50	12.50	12.50	..	..	..	..	10.00	10.00	10.00	10.00	
Sardine meal	2.50	2.50	2.50	..	..	..	..	2.50	5.00	7.50	10.00	
Condensed fish solubles	..	2.00	4.00	..	..	2.00	4.00	..	..	..	..	
Glycine	..	..	..	..	1.00	..	..	..	..	..	..	
Soybean meal	..	..	..	34.50	34.50	34.50	34.50	..	..	..	..	
Corn gluten meal	20.00	20.00	20.00	..	..	..	..	6.50	5.67	4.83	4.00	
Cottonseed meal	..	..	..	..	..	..	..	6.50	5.67	4.83	4.00	
Linseed meal	..	..	..	..	..	..	..	6.50	5.67	4.83	4.00	
Flaydry	2.00	2.00	2.00	..	..	..	..	2.00	2.00	2.00	2.00	
Ladpro 100 D	1.60	1.60	1.60	..	..	..	..	1.60	1.60	1.60	1.60	
400 D fish oil	..	..	..	0.50	0.50	0.50	0.50	..	..	..	..	
Ground limestone	2.50	2.50	2.50	1.00	1.00	1.00	1.00	2.50	2.50	2.50	2.50	
Steamed bone meal	..	..	..	2.00	2.00	2.00	2.00	..	..	..	..	
Salt with I <sub>2</sub> and Mn	1.00	1.00	1.00	0.50	0.50	0.50	0.50	1.00	1.00	1.00	1.00	
Additional vitamins	..	..	..	(See footnote <sup>1</sup> )								
Protein % (calculated)	25.7	26.3	26.9	20.5	21.4	21.1	21.7	23.2	23.7	24.2	24.7	
Lysine % (calculated)	0.81	0.85	0.89	1.04	1.04	1.08	1.12	0.82	0.89	0.95	1.02	
No. of poult	10	10	10	10	10	9	9	7	7	7	7	
Average weight at 5 weeks	355	384	387	298	295	420	393	220	340	336	350	
Achroma score	4	3.5	3.5	2	2	1	0	3	3	2	1	

<sup>1</sup> To diets 4443 to 4446, inclusive, the following crystalline vitamins were added per pound: 1.2 mg riboflavin, 68.0 mg nicotinic acid, and 3.4 gm choline chloride.



A reasonable rate of growth is required to bring out the abnormality. If growth is below a critical limit, the lysine requirements are apparently reduced to the point where sufficient lysine is available for pigmentation.

Lysine is also seen to be a growth factor. This confirms the conclusion that the syndrome described is actually a deficiency. In all but a few cases the addition of either a natural source of lysine or of crystalline lysine did stimulate growth as well as prevent the pigmentation failure.

There are a few discrepancies in the growth rate which are not fully explained. The growth on diet 4213 was relatively poor (table 1). However, the average weight shown is influenced considerably by an unusual number of very small poults. If these were eliminated, the average weight of the birds on diet 4213 would be fully up to the highest weights obtained with other diets in this trial.

Calculations of the lysine content of the various rations are based upon data of Almquist ('45) and Block and Bolling ('45). With a few exceptions, chiefly those summarized in table 3, the incidence of the white feather syndrome varied inversely with the lysine content of the ration. Diets which contained 1.1% to 1.2% of lysine seemed to meet the poults' requirements for growth and pigmentation. These figures are above the recommended nutrient allowances for chickens (Cravens et al., '44). It seems apparent that the turkey poult requires considerably more lysine than does the chick. Similar differences have been noted in vitamin requirements.

When poults were placed on a lysine deficient diet, the white feather syndrome became apparent at about 2 weeks of age. The abnormal condition reached a maximum at about 5 weeks of age, and then gradually receded. Even when the poults were kept on diet 4215, the normal bronze pigmentation had nearly completely replaced the white by the time the poults were 10 weeks of age. This is somewhat more rapid disappearance of the syndrome than was noted by Funk and Kempster ('40).

As would be predicted from the lysine content, fish solubles and fish meal showed little protective value. Unless higher than usual levels of these ingredients are used, they are not especially effective in preventing the white feather syndrome. They do, however, show a growth stimulating value which may be due to other factors. Variations in the fiber or calcium content of the ration, and additions of glycine and calcium pantothenate, had no apparent influence on the incidence of the white feather syndrome.

Some of the poults on these diets also developed a curled feather condition similar to the symptoms of vitamin B<sub>10</sub> deficiency described by Briggs, Luckey, Elvehjem and Hart ('44). This condition did not parallel the pigmentation failure, and may have indicated another deficiency in the rations used.

#### SUMMARY

A feather pigmentation failure was observed in bronze poults raised in confinement on diets containing a high percentage of certain vegetable protein concentrates. The syndrome was prevented by adding crystalline lysine to the diet, or by substituting protein concentrates high in lysine. The data indicates that the diet must contain approximately 1.1 – 1.2% of lysine for normal feather pigmentation and to permit optimum growth of poults.

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# SELF SELECTION OF DIET

## I. SELECTION OF PURIFIED COMPONENTS<sup>1</sup>

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TWO FIGURES

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On the assumption that preferences for foods are indicative of nutritional requirement and therefore physiologic need, several attempts have been made to determine the effect of varying physiologic requirement on the voluntary intake of purified or partially purified food components by the rat. Under certain conditions, successful choice has been reported (Richter et al., '38a, '38b, '38c, '39, '41a, '41b; Clark and Clausen, '43; Cahill et al., '43). Other investigators have found a limited ability of rats to select proper foods (Kon, '31; Warkentin et al., '43).

The present experiments were designed as an attempt to clarify the role of nutrition in the self selection behavior of rats.

### EXPERIMENTAL METHODS

Albino rats of a mixed strain were weaned at 21 to 25 days of age and put in individual cages with four identical cups in a rack, all filled with the standard diet.<sup>2</sup> Vitamins were

<sup>1</sup> Contribution No. 581 of the Department of Chemistry, University of Pittsburgh. Aided by a research grant of the Buhl Foundation.

<sup>2</sup> The standard diet consisted of 24% purified casein (Labco "vitamin-free"), 4% salts (Jones and Foster, '42), 10% hydrogenated fat (Primex), and 62% sucrose.

supplied daily in the form of pills.<sup>3</sup> This diet and supplement have given satisfactory growth in all experiments in this laboratory in which they were used.

The amount of food eaten from each cup was recorded daily, and the cups were then interchanged in a predetermined random manner. After a 3-week control period, the standard diet was replaced by sucrose, Primex, casein, and salts, each in an individual cup. The amount of each component was recorded daily for 3 weeks, and the cups interchanged in the same random manner as before.

### RESULTS

Three types of eating behavior were apparent in the 3-week control period. These were: (1) Position eating — The animals preferred to eat from the two cups on the end, as opposed to the two in the middle. As individuals, they usually ate more from either the right or left end cup, and more animals ate from the right than from the left end cup. (2) Repetitious eating — The animals had a tendency to eat from the cup they had eaten from before, in spite of its position or change of position. (3) Random eating — Certain animals ate at random from the cups. This tendency could very properly be considered a lack of the first two types of behavior.

As would be expected from general knowledge of animal behavior, the proportion of animals which showed one type of eating behavior to the exclusion of the others was small. Most animals therefore had tendencies toward all three. A summary of the eating habits during the control period of eighty-seven animals used in this study is shown in table 1. The division into two groups, A and B, is based on growth performance during the experimental period. Group A lost weight and group B gained weight during the latter period.

<sup>3</sup> Each pill contained approximately: 60  $\mu$ g thiamine hydrochloride; 120  $\mu$ g riboflavin; 90  $\mu$ g pyridoxine hydrochloride; 150  $\mu$ g calcium pantothenate; 10 mg choline chloride; 1 mg  $\alpha$ -tocopherol; 55 IU Vitamin A and 11 IU Vitamin D, both as 0.001 ml Natola in a dextrin-powdered sugar base. One pill was offered each rat daily throughout the experiment and, after the first 2 or 3 days, it was consumed avidly.

During the 3-week experimental period all possible types of eating behavior were observed. A few animals ate only sucrose or only fat until they died; some animals alternated from one to the other; some animals ate no protein; others for a fairly long period ate protein exclusively. The only generalization that could be drawn was that no animal disliked all of the choices, and accordingly no indications of caloric deficiency were observed. The only differences of choice in the two sexes were attributable to differences in growth rate and to total amount of food eaten.

TABLE 1  
*Eating behavior of rats during 3-week control period.<sup>1</sup>*

GROUP	NO. OF ANI- MALS	GROWTH	FOOD EATEN	% TOTAL FOOD EATEN FROM POSITION <sup>2</sup>			
				(a)	(b)	(c)	(d)
		gm	gm				
A ♂	20	49.5 ± 3.3	108.4 ± 5.4	22.4 ± 1.2	20.4 ± 2.1	24.2 ± 2.1	33.0 ± 2.9
♀	14	45.0 ± 3.6	100.0 ± 5.6	23.5 ± 2.6	19.8 ± 1.9	19.3 ± 1.8	37.6 ± 4.0
All	34	47.0 ± 2.5	105.0 ± 3.9	22.8 ± 1.3	20.2 ± 1.4	22.2 ± 1.5	34.9 ± 2.4
B ♂	28	66.4 ± 2.6	133.8 ± 3.8	27.8 ± 2.3	20.5 ± 1.4	21.5 ± 1.0	28.9 ± 1.6
♀	25	59.7 ± 2.4	126.9 ± 3.4	27.2 ± 2.6	21.3 ± 1.5	21.5 ± 1.1	30.2 ± 1.8
All	53	63.2 ± 1.8	130.5 ± 2.6	27.5 ± 1.7	20.8 ± 1.0	21.5 ± 0.9	29.5 ± 1.8
Total	87	56.9 ± 1.7	120.5 ± 2.6	25.7 ± 1.2	20.6 ± 0.8	21.8 ± 0.8	31.6 ± 1.2

<sup>1</sup> All data in terms of mean and standard error of the mean.

<sup>2</sup> Position (a) was to the animals left while eating.

Because of the marked variability in eating behavior and growth response, the data obtained were difficult to present quantitatively. The frequency distributions of fat, protein, and carbohydrate in terms of per cent by weight of food eaten or per cent of total calories did not approach the normal frequency curve, and it was believed that calculated averages and standard errors would be almost meaningless in these cases. However, a histogram of weight of fat eaten approached the normal distribution; weight of protein and salt gave bimodal distributions, and weight of carbohydrate gave a skewed unimodal distribution (fig. 1). The situation was

further clarified by the division of the animals into two groups on the basis of growth. (Group A lost weight during the experimental period; group B gained weight). Each group showed a frequency distribution sufficiently near the normal to allow the calculation of average values and their standard errors for weights of fat, protein, and salt eaten as well as for weight change and total calories eaten (table 2). Both groups

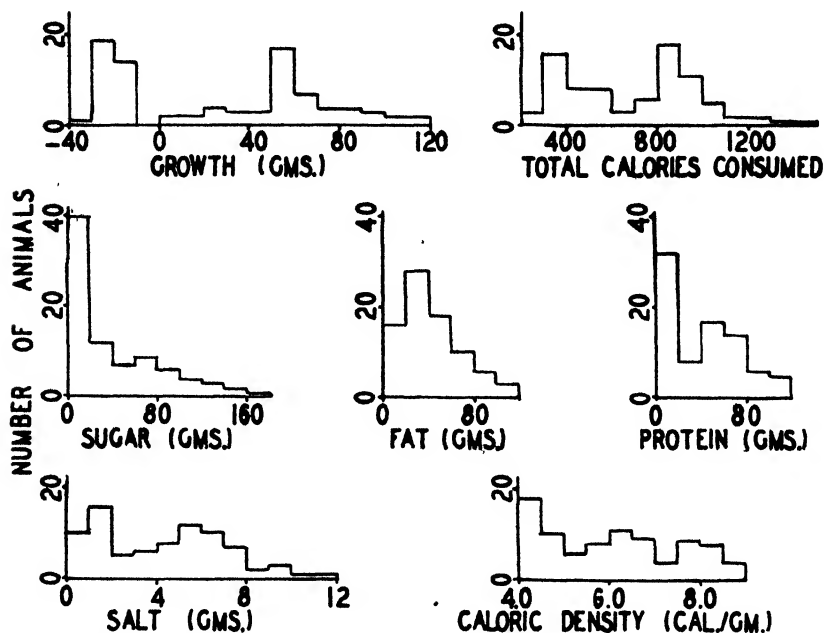


Fig. 1 Distribution of growth and eating habits of rats during a 3-week period of self selection.

showed the same skewed distribution for weight of carbohydrate eaten as is shown in figure 1, and the average figure for weight of carbohydrate presented in table 2 is of doubtful significance. A calculated standard error of weight of carbohydrate would obviously be meaningless under the circumstances.

It will be noted from table 1 that those animals which grew on the selection experiment also grew significantly more during the control period.

Histograms of calculated caloric density (total cal./total weight of food) of diets selected by both group A and group B showed bimodal frequency distributions (fig. 2). A somewhat arbitrary division of the groups on the basis of caloric density resulted in groups which ate (aside from protein and salts) primarily fat or primarily carbohydrate for the balance

TABLE 2

*Average selections of animals during 3-week experimental period.*

GROUP	NO. OF ANI- MALS	CHANGE IN BODY WT.	TOTAL CALORIES EATEN	PROTEIN EATEN	FAT EATEN	SALT EATEN	CARBO- HYDRATE EATEN <sup>2</sup>
		gm		gm	gm	gm	gm
A ♂	20 <sup>1</sup>	-21.8 ± 1.3	421 ± 28	1.4 ± 0.5	38.3 ± 3.9	0.7 ± 0.1	18.1
♀	14 <sup>1</sup>	-19.8 ± 0.9	382 ± 26	1.3 ± 0.4	30.2 ± 4.5	1.3 ± 0.3	29.8
All	34 <sup>1</sup>	-21.0 ± 0.8	403 ± 19	1.3 ± 0.4	35.0 ± 3.0	0.9 ± 0.2	22.9
B ♂	28	66.6 ± 5.8	920 ± 45	58.5 ± 5.2	50.4 ± 6.9	5.4 ± 0.4	58.1
♀	25	50.4 ± 3.3	829 ± 29	62.0 ± 5.2	40.1 ± 4.2	5.4 ± 0.4	55.0
All	53	58.9 ± 3.6	877 ± 28	60.1 ± 3.7	45.5 ± 4.2	5.4 ± 0.3	56.7

<sup>1</sup> Three males and two females died in group A, presumably due to lack of protein in the diet.

<sup>2</sup> For discussion of significance of this figure, see text.

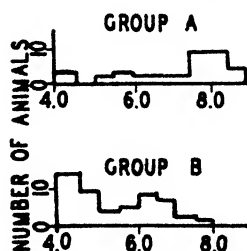


Fig. 2 Distribution of caloric densities of self selected diets.

of their diet. The subdivided groups (table 3) did not differ greatly in other respects. The differences that appear in weight change and total calories of groups B<sub>1</sub> and B<sub>2</sub> in table 3 are in part due to the uneven distribution of the sexes in the two groups.

Rats from fourteen complete litters of varying size were used in this experiment. Table 4 shows the sex distribution and numbers of animals in groups A and B of each litter.



The probability that an arrangement, as improbable as the group distribution, could result from the litter being a random sample of all animals used was calculated.<sup>4</sup> In table 4 it is seen that seven of fourteen litters have probabilities

TABLE 3  
*Relation of caloric density of self-selected diets to growth,  
calories and food choice.*

		G R O U P			
		A <sub>1</sub>	A <sub>2</sub>	B <sub>1</sub>	B <sub>2</sub>
Number of animals:	♂	16	5	16	12
	♀	7	6	10	15
Caloric density		8.0 ± 0.1	5.4 ± 0.3	6.3 ± 0.1	4.5 ± 0.1
Change in body weight (in gm)		— 21.8 ± 1.0	— 19.3 ± 1.3	65.5 ± 5.1	52.7 ± 3.3
Total calories eaten		404 ± 25	400 ± 29	917 ± 38	839 ± 42
Protein eaten (in gm)		16 ± 0.5	0.9 ± 0.3	57.0 ± 3.7	63.1 ± 6.3
Fat eaten (in gm)		41.8 ± 2.5	19.7 ± 5.1	70.9 ± 3.8	21.1 ± 2.5
Carbohydrate eaten (in gm)		7.4 ± 1.2	57.3 ± 5.7	12.7 ± 1.4	99.0 ± 7.2
Salt eaten (in gm)		0.8 ± 0.2	1.1 ± 0.4	5.5 ± 0.3	5.3 ± 0.5

less than 0.22. Since the probability of the latter occurring due to random sampling is 1 in 50 (0.020), litter mates must tend to have the same appetites. It is interesting, however, that no litter of four or more animals failed to have members in both groups.

<sup>4</sup> The probability of any one animal being in group A is  $34/87 = .39$ . The probability  $P_A$  that  $X$  animals in a random sample of size  $n$  will be in group A is:

$$P_A = \frac{n(n-1)}{1 \cdot 2 \cdot 3} \cdots \frac{(n-X+1)}{(n-X)} \quad (.39)^X \quad (.61)^{n-X}$$

The probability that an arrangement as improbable as  $X$  of  $n$  animals in group A could result from random sampling is  $P_A$  plus the sum of the probabilities of all less probable arrangements. The probability  $P_B$  of seven of fourteen litters having a probability of less than 0.22 obviously:

$$P_B = \frac{14 \cdot 13 \cdot 12 \cdot 11 \cdot 10 \cdot 9 \cdot 8}{1 \cdot 2 \cdot 3 \cdot 4 \cdot 5 \cdot 6 \cdot 7} \cdot (.22)^7 \cdot (.78)^7 = 0.015$$

The sum of the probabilities of less probable arrangements of fourteen litters is 0.005, and therefore the probability that an arrangement of fourteen litters could be as improbable as that shown in table 4 because of random sampling is 0.020.

TABLE 4

*Litter distribution of animals on self selection.*

LITTER	NUMBER OF ANIMALS				PROBA- BILITY <sup>1</sup>	LITTER	NUMBER OF ANIMALS				PROBA- BILITY <sup>1</sup>
	(1)	(2)	(3)				(1)	(2)	(3)		
	♂	♀	Group A	Group B			♂	♀	Group A	Group B	
D	2	2	2	2	0.646	K	6	4	3	7	0.750
C	3	3	5	1	0.037	L	3	3	1	5	0.415
E	4	2	1	5	0.415	M	3	3	4	2	0.217
F	1	0	1	0	0.390	N	6	2	1	7	0.165
G	6	4	8	2	0.017	O	4	5	1	8	0.177
H	0	1	0	1	1.000	P	3	2	4	1	0.079
I	3	3	2	4	1.000	Total	48	39	34	53	
J	4	5	1	8	0.177						

<sup>1</sup> Probability that a group distribution as improbable as that in column (2) could arise from random sampling.

## DISCUSSION

The pronounced variability and atypical frequency distributions of the choices of the rats in this experiment cannot be too strongly emphasized. These characteristics prevent the calculation of averages and standard errors of appetites of small numbers of animals, and limit the usefulness of the self-selection method to large groups.

Under present conditions, the following conclusions can be reached:

1. Rats either do or do not like casein; if they like it, they eat an average of 3 gm per day and grow well; if they do not, they eat less than 0.1 gm per day, lose weight, and die within a short period.

2. Rats have a uniform appetite for hydrogenated fat which varies only slightly with the growth rate, and therefore with the total calories eaten.

3. A large proportion of rats eat very little sucrose, while only a few eat it in large amount.

4. Rats which grow eat about 0.25 gm of salts per day; rats which do not, eat 0.05 gm per day.

5. Rats which like casein grow significantly better on a standard diet than do rats which dislike casein.

6. Litter mates tend to have similar appetites, indicating the influence of hereditary factors on choice.

Two necessary criteria that an appetite be based on a dietary need are: (a) The need must be accompanied by an appetite. (b) A change in need must be accompanied by a change in appetite. These are not sufficient criteria, however, to prove the point because the animal may learn by experience that a particular choice gives him a greater feeling of satisfaction, while obviously he is unable to recognize a dietary need as such.

It is in the light of these two criteria that the dietary significance of this experiment becomes most apparent. Since all the rats had a need for protein, and only fifty-three out of eighty-seven had an appetite for it, there does not appear to be a relation between need and appetite in this case. It is even possible that the appetite for protein of the animals in group B may be based on a trivial factor such as taste, consistency, or desire for variety. The widely varied appetites for fat and carbohydrate seem to have no basis in dietary need.

The appetite for salts does seem to be related to need. With few exceptions, the animals which exhibited growth selected salts and those which did not ate very little. There was no evidence of growth retardation due to low intake of salts. Similarly, the appetite for calories seemed to be related to need. No animal failed to grow because of lack of calories, and no animal lost weight unusually rapidly because of failure to eat. The type of food eaten to supply calories, aside from protein, had little effect on total calories eaten, as shown in table 3, and the only appetite which affected growth was that for protein.

The most probable conclusions under the conditions of this experiment appear to be: (a) The only nutritional requirements related to eating behavior are need for salts and need for calories. (b) The form in which calories are eaten is independent of need. (c) The need for salts and calories is

determined by the protein intake, which, of itself, is independent of need.

Finally, it is common knowledge that dietary requirements of a group of animals, like all physiologic measurements, can be described as a mean plus or minus a small standard error. The wide variability of the appetites here demonstrated can be considered as an additional indication that they are not based on dietary needs, for the latter show much less variability.

The fact that rats which are able to make a successful choice also grow better on a standard diet is of special interest. If a similar correlation exists in the case of hogs, the explanation of the good growth of these animals on self-selection feeding may be the fact that they have been bred for many generations for growing ability.

The results here presented are appreciably different from those presented in several of the studies mentioned above. Possible explanations of differences in these and earlier results may be as follows: (1) In some reports, the number of animals was too small to draw any significant conclusions. (2) Rats of a different strain were used. (3) Some of the choices presented were not purified foods. For example, a large portion of the protein requirement of rats in certain experiments has been provided by yeast.

#### SUMMARY

1. The appetites of eighty-seven normal rats, allowed their choice of sucrose, casein, hydrogenated fat, and salts, are described. Thirty-four of these animals failed to grow.

2. The animals could be separated into two groups on the basis of their appetites for casein.

3. The appetites of these animals, aside from that for salts and calories, showed no apparent relation to physiologic or nutritional need.

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# VARIABILITY IN THE CALCIUM METABOLISM AND CALCIUM REQUIREMENTS OF ADULT HUMAN SUBJECTS<sup>1</sup>

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Variability is the most prominent characteristic of living beings. Variability among the individuals of the same zoological (or botanical) class or subdivision is spoken of as individuality, and the biological basis of individuality has been considered in great detail recently by Loeb ('45). But in the same individual, variability operates in functional behavior from time to time, even under the same environmental conditions, insofar as these conditions can be controlled in biological experimentation. The universality of variation, both morphological and physiological, among and within individual organisms, complicates the planning of experimental investigations, the interpretation of the results secured, and their application to the practical problems of life.

The prevalence of variation in every type of biological study necessitates in most cases the application of statistical analysis in order to distinguish the changes in form or function that may result from changes in conditions deliberately imposed in the plan of the experiment, from the changes in form or function that occur regardless of these changed conditions

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due to the uncontrolled factors in the experiment and to the individuality of the subject organisms.

In physiological and biochemical studies on man and laboratory animals, the prevalence of functional variability under conditions as well controlled as is feasible at present has been pointed out from time to time. The publications of Rietz and Mitchell ('10), Grindley and Mitchell ('17), Bauer, Allbright and Aub ('29), McCance and Widdowson ('42, '43), and Macy ('42), among many others, may be cited in this connection. The variation in biological assays of pharmaceutical products and the service of statistical methods of analysis in dealing with the situation have been ably discussed by Bliss ('41).

However, in nutritional research, the fact that variation in experimental data must be considered and measured for purposes of description and valid interpretation is not always recognized. The simplest mathematical manipulation, the computation of an average, is often the only statistical method used in reducing data to a form from which conclusions are deduced. The process of adding individual data together and dividing by their number, to obtain a value that generally is not identical with any of the measurements actually made, is carried out apparently in a routine way, with little consideration of the consequences of substituting an average for the data from which it was derived, or of its implications. Food composition tables that are so important in practical nutrition in implementing nutritional principles and generalizations, contain only average analyses. Such an abbreviated presentation, often of a rich volume of data, fails to reveal the reliability of different foods as sources of nutrients. It misleads the user of such tables to class together, as a source of calcium for example, a reliable food such as milk with an utterly unreliable food, such as lettuce, merely because their average contents of calcium for the particular samples analyzed happen to be nearly the same.

Similarly, in the presentation of nutritive requirements, averages only are given in the majority of cases. One common use to which such averages are put is the evaluation of the

nutritive status of individuals and populations whose nutritive intake has been more or less accurately estimated. But with no information as to the variability in requirements, the significance of deviations between the average requirements and the estimated intakes cannot be judged on any rational basis. Whether the deviations are within the range of normal requirements, or whether they indicate definite over- or under-nutrition cannot be decided, although unfortunately decisions are often made nevertheless. Pett ('43, '45) has ably remonstrated against such a use of requirement data and has strongly advocated the determination of variation in nutritive requirements, as well as mean requirements. Fertig ('43) and Dann and Darby ('45) have presented similar arguments. Kline and Eheart ('44) have reported extreme variations in the ascorbic acid requirements for saturation, even in a small group of women subjects, while Steggerda and Mitchell ('41) have observed a variability of about 20% (coefficient of variation) in the calcium requirements of a small group of men.

It will be the purpose of this paper to report the results of calcium metabolism studies on a group of nineteen men with particular reference to the variation among and within individuals with reference to the paths of excretion and the utilization of ingested calcium and to the indicated requirements of dietary calcium for the maintenance of calcium equilibrium.

#### EXPERIMENTAL PROCEDURES

There were nineteen subjects in the experiments reported in this and the following paper, which extended over a period of 5 years. Their average age, weight, and height are recorded in table 1, together with estimated surface areas and basal metabolism.

The general plan of the experiment in testing the utilization of the calcium in various milk products and calcium salts was to place each subject upon a basal diet containing on the average 229 mg of calcium daily. This diet contained sufficient food energy to maintain body weight and, with daily supplements of vitamins A and D, it was presumably adequate in all



respects but calcium. After 4 or 5 days on a constant intake of food, collections of feces and urine were made in successive 4-day periods until it was apparent that the subject was adjusted to this low level of calcium nutrition. Carmine was used as a feces marker.

In succeeding metabolism periods, supplements of milk products and calcium salts were added to the basal diet in

TABLE 1  
*Description of experimental subjects.*

SUBJECT	AGE	BODY WEIGHT	HEIGHT	BODY SURFACE <sup>1</sup> AREA	BASAL METABOLISM <sup>2</sup>
	<i>years</i>	<i>kg</i>	<i>cm</i>	<i>m<sup>2</sup></i>	<i>Cal</i>
Steg	39	81	180	2.01	1817
Ne	28	74	184	1.96	1815
Cl	27	70	176	1.86	1727
Fo	25	67	171	1.78	1675
Step	25	83	171	1.95	1895
Has	25	72	204	2.09	1909
Ve	22	73	186	1.97	1852
Sc	22	87	186	2.12	2045
McB	24	64	181	1.82	1690
Bc	28	67	169	1.77	1644
Te	26	67	179	1.84	1708
Sn	23	72	181	1.92	1807
McC	20	63	179	1.80	1694
Sw	20	65	176	1.80	1706
On	20	67	173	1.80	1719
To	29	62	167	1.70	1559
Hal	19	66	166	1.73	1677
Le	17	63	166	1.70	1650
Ro	24	70	186	1.93	1797

<sup>1</sup> Computed by the formula of DuBois and DuBois ('16).

<sup>2</sup> Estimated by the Harris-Benedict (Carpenter, '39) formula.

amounts to provide enough calcium for approximate equilibrium. The milk products tested included pasteurized whole milk, dried defatted milk, dried whole milk, homogenized milk, a commercial soft curd milk prepared by the process of base exchange (Lyman et al., '33), milk heated to 160°F. for 30 minutes and milk with varied supplements of sodium alginate (2%), potassium or sodium citrate, citric acid (0.27%),

and orange juice. The calcium salts used were the citrate and the gluconate. During those periods involving milk supplements, small adjustments were made in the basal diets to equalize the energy intakes in basal and test periods. All experimental periods consisted of a succession of 4-day collection periods continued until it was evident that an adjustment to the experimental diet had occurred over at least 12 days, and generally 20 days or more. Results of a 4-day period at the beginning of a test that indicated incomplete adjustment were not included in the average.

Further details of the experimental procedure are contained in a previous article (Steggerda and Mitchell, '39).

#### EXPERIMENTAL RESULTS

The average results for each subject on the low-calcium basal diet are summarized in table 2. The averages include all

TABLE 2  
*Calcium metabolism at low intake levels.*  
*Averages expressed in milligrams per day.*

SUBJECT	PERIODS	TOTAL DAYS	CALCIUM INTAKE	CALCIUM IN FECES	CALCIUM IN URINE	CALCIUM BALANCE
Ne	4	84	229	268	60	— 99
Fo	4	60	236	284	79	—127
Steg	11	192	204	216	78	— 90
Cl	2	32	198	204	189	—195
Step	3	56	217	274	77	—134
Has	2	40	208	206	86	— 84
Ve	1	24	221	182	144	—105
Sc	2	44	248	273	112	—137
Ro	1	16	201	232	177	—208
Te	1	20	216	307	76	—167
Be	1	20	213	274	65	—126
Sn	5	104	245	247	134	—136
McC	1	20	261	280	237	—256
Sw	1	20	261	150	171	— 60
On	1	20	261	233	100	— 72
To	5	108	195	89	210	—104
Hal	1	20	225	185	175	—135
Le	1	20	225	196	28	+ 1
McB	1	20	281	256	172	—147

of the low-calcium periods for each man, ranging from 1 to 11, and from 16 to 192 days. For twelve of the nineteen subjects, the fecal calcium exceeded the intake, and for seventeen of the subjects, the fecal calcium exceeded the urinary calcium. For eight of the subjects, the urinary calcium averaged less than 100 mg daily, but for six of them, it approximated or exceeded the fecal calcium. These relations testify to the prominence of individuality within this group of men with reference to path of excretion of ingested calcium. The calcium balances are not inversely correlated with the fecal calcium, and for this and other reasons to be developed in the following paper, the authors do not believe that fecal calcium is entirely, or even largely, composed of ingested calcium that has escaped absorption from the gastro-intestinal tract.

The standard deviation of the 212 4-day calcium balances about the means for their respective subjects was 46.3 mg per day. This value measures the functional variability of the subjects while subsisting on essentially the same low-calcium diet. It does not include the variation existing among different subjects, a variation that seems impossible to assess surely from these data, because of the lack of any rational method of correcting calcium balances either for differences in calcium intake or for differences in size of subject. If these differences may be neglected as possessing only minimal effects within the ranges of calcium intake and body size prevailing, the standard deviation of the nineteen average calcium balances is 57.2 mg per day, the average balance being — 125 mg per day.

These standard deviations express in a quantitative fashion the great variation existing within the same individual, but particularly among different individuals, in the disposal of a low level of dietary calcium, in the utilization of dietary calcium in the sparing of body calcium, and possibly in the rate of the endogenous excretion of calcium. It will be noted in particular that subject Le for a period of 20 days was in calcium equilibrium on an intake of 225 mg per day. In contrast, subject McC, somewhat larger than Le but with a calcium intake also 36 mg larger daily, exhibited a negative

balance of 256 mg daily, again over a 20-day period. These extremes in balance deviate from the mean balance for all subjects by only slightly more than twice the standard deviation of 57 mg, and hence they must be considered as resulting merely from a random combination of uncontrolled factors operating throughout the entire group of subjects.

The balance data secured at the higher levels of calcium intake, averaging 545 mg daily, have been pooled together for each subject, regardless of the nature of the supplemental calcium. We have felt justified in doing this, because an intensive study of the data has not revealed any statistically significant differences in utilization of the different supplements of calcium, that is, differences in their sparing effects on body calcium, possibly due in part to the great variation in metabolic performance of the human organism. In support of this statement, the following average percentage utilizations<sup>2</sup> for the various supplements may be cited:

Milk alone, 34% in 24 tests; milk + sodium citrate or citric acid, 34% in 13 tests; milk heated to 160°F. for 30 minutes, 35% in 4 tests; milk + sodium alginate, 29% in 3 tests; dried milk, whole or defatted, 19% in 6 tests; milk + orange juice, 34% in 4 tests; soft curd milk (base-exchange), 29% in 9 tests; homogenized milk, 24% in 4 tests; calcium citrate, 30% in 5 tests; and calcium gluconate, 25% in 4 tests.

It may be thought that the calcium in the dried milk preparations was definitely less well utilized than that of the other supplements. However, the average of 19 is derived from six determinations, two of which were abnormally low, one, in fact, being negative. If these two values are disre-

<sup>2</sup> The calculation of a percentage utilization of calcium may be illustrated from the following data for subject Step:

Low-calcium period	217 mg Ca intake	-134 mg Ca balance
Same plus milk and Na citrate	458 mg Ca intake	- 57 mg Ca balance
Differences	241 mg Ca	77 mg Ca

$$(77 \div 241) \times 100 = 32\% \text{ utilization.}$$

garded, the average becomes 28. The results on the soft curd (base-exchange) milk confirm those of Hess et al. ('40) on infants.

Considering all supplemental sources of calcium as statistically indistinguishable in adult human metabolism, the standard deviation of the 308 4-day calcium balances at the higher level of intake, about the means for their respective subjects, was 59.5 mg per day, a value that measures the functional variability of the subjects while subsisting on a level of calcium approximating that required for equilibrium. A similar standard deviation relating to the percentage utilizations is 9.85%. The latter value refers, however, not to 4-day periods, but to the periods averaging about 20 days in length, employed in the determination of calcium utilization. Evidently, with a standard deviation of this magnitude, it would be difficult to establish the reality of a difference in calcium utilization in the same subject of less than 18.5 percentage units ( $P=.030$ ), using a test period of 20 days. With a number of subjects, rather than one, this critical difference would be diminished in proportion to the square root of the number used.

The pooled data for each of the nineteen subjects for calcium metabolism at the higher level of intake, together with individual estimates of calcium utilization and of calcium requirement for equilibrium, are summarized in table 3. The average utilization of calcium, computed according to footnote 2, is 31.6. This value would relate to a diet in which about 60% of the calcium is provided by milk, or by calcium salts approximately as well utilized in adult metabolism as the calcium of milk. The standard deviation of individual utilization percentages is 7.36, and the coefficient of variations, 23.3%. The mean value of 31.6% is very nearly the same as that previously reported (Steggerda and Mitchell, '41) for a group of nine men, three of whom were included in the present study. The average calcium utilization reported in the prior study was 29%.

TABLE 3  
*Calcium utilization and indicated requirements at borderline levels of intake.*

SUBJECT	NUMBER OF PERIODS	TOTAL	CALCIUM REQUIREMENTS PER DAY						
			CALCIUM INTAKE	CALCIUM BALANCE	CALCIUM UTILIZATION	Total	Per kg body weight	Per m <sup>2</sup>	Per basal cal
		days	mg	mg	%	mg	mg	mg	mg
Ne	11	208	649	- 18	19	739	9.99	377	.407
Fo	10	188	634	+ 17	36	588	8.78	330	.351
Steg	14	256	573	+ 5	26	554	6.84	276	.305
Cl	8	160	652	- 48	32	800	11.43	430	.463
Step	1	20	458	- 57	32	636	7.66	326	.336
Has	1	20	430	- 50	16	739	10.26	354	.387
Ve	2	40	589	- 4	27	604	8.27	307	.326
Sc	4	84	668	- 19	28	736	8.46	347	.360
Ro	1	20	516	-111	31	876	12.51	454	.487
Te	1	20	498	- 67	35	686	10.24	373	.402
Be	1	20	532	- 36	28	660	9.85	373	.401
Sn	7	152	600	- 44	26	771	10.71	402	.427
McC	1	28	646	-126	34	1018	16.16	566	.601
Sw	3	72	438	+ 17	43	399	6.14	222	.234
On	1	28	465	+ 20	45	421	6.28	234	.245
To	3	60	421	- 26	35	492	7.94	289	.316
Hal	2	40	568	- 28	31	658	9.97	380	.392
Le	2	40	408	+ 66	35	222	3.52	131	.135
McB	2	40	619	- 9	41	641	10.02	352	.379
Means		20	545	- 25	31.6	644	9.21	343	.366
Standard deviations					7.36	181	2.71	93.8	.101
Coefficients of variation, pct.					23.3	28.1	29.4	27.3	27.5

The indicated calcium requirements, computed as in a previous publication (Steggerda and Mitchell, '41)<sup>3</sup> averaged 644 mg per man per day, with a standard deviation of 181 mg. These daily requirements were each expressed per kilogram of body weight, per m<sup>2</sup> of body surface, and per basal calorie in an attempt to eliminate the effect of variable body size. The mean values obtained were 9.21 mg of calcium per kilogram of body weight, 343 mg per m<sup>2</sup>, and 0.366 mg per basal calorie. However, it is worthy of note that the coefficients of variation were the same (about 28%) for all practical purposes, whether the values per subject, or per unit of weight, surface, or basal metabolism are considered. In other words, these attempts to eliminate the effect of variable body size were unsuccessful. We do not think, with Leitch ('37), that this means calcium requirements bear no relation to body size. This to us is inconceivable. Rather, it probably means that other factors are so much more potent in causing variation in calcium metabolism as to completely obscure the effect of variable body size.

The calcium requirements summarized in table 3 may be compared with others obtained in a similar fashion and relating also to diets whose calcium contents are derived predominantly from dairy products. Such comparisons are assembled in table 4.

The values for the average total daily requirement of calcium in the four experiments compared exhibit a truly remarkable agreement, being grouped closely about the grand average of 653 mg. Reduced to the per kg and to the per m<sup>2</sup> basis, this total requirement becomes 9.99 mg and 365 mg, respectively. In good agreement also is the average of 9.75 mg of calcium per kilogram of body weight per day computed by Mitchell and Curzon ('39) from selected experiments in the older literature. As in the experiment reported in this

<sup>3</sup> An illustration of the method of computation may be taken from subject Ne. On the basal diet containing 229 mg of calcium daily, the average calcium balance was -99.0 mg. With an average utilization of calcium of 19.4% for this man, the negative balance would be wiped out on feeding  $99.0 \div .194 = 510$  mg additional calcium, or a total of  $229 + 510 = 739$  mg daily.

TABLE 4

*Comparison of average daily calcium requirements in different experiments, determined by the same method with diets containing 53 to 71% of their calcium in milk or equivalent products.*

AUTHORITY	NUMBER OF SUBJECTS	TOTAL		PER KG		PER M <sup>2</sup>	
		Mean mgs	Coefficient of variation <sup>1</sup>	Mean mgs	Coefficient of variation <sup>1</sup>	Mean mgs	Coefficient of variation <sup>1</sup>
			%		%		%
Steggerda and Mitchell ('41)	9 <sup>2</sup>	657	17.8	9.55	21.6	357	19.1
Breiter et al. ('41)	7 <sup>3</sup>	662	23.1	10.70	16.9	387	16.9
This report	19 <sup>2</sup>	644	28.1	9.21	29.4	343	27.3
Unpublished <sup>4</sup>	8 <sup>5</sup>	664	18.7	11.61	15.5	408	15.0
Total population <sup>6</sup>	43	653	22.5	9.99	23.1	365	21.8

<sup>1</sup> The coefficient of variation is the standard deviation expressed as a percentage of the mean.

<sup>2</sup> All men.

<sup>3</sup> Four women, 3 men.

<sup>4</sup> Calcium metabolism studies carried out jointly by the Division of Foods and Nutrition and the Division of Animal Nutrition, University of Illinois.

<sup>5</sup> All women.

<sup>6</sup> Disregarding the variation among the mean results in each experiment.



paper, the variation in individual requirements is about 22%, and is essentially the same for all three methods of expression.

The average daily calcium requirement for equilibrium of adult humans of 10 mg per kilogram seems to be well established by the experiments cited in this paper for individuals of the economic and social class studied, i.e., college students and staff members. It would seem to be a safe value to use in the planning of diets containing an average proportion of calcium from dairy products for groups of individuals. For such cases, there is no good reason for boosting the value of any arbitrary "margin of safety," any more than there is for a requirement of food energy. Apparently, the only good reason for incorporating a "margin of safety" in one case but not in the other, is that, with calcium, as contrasted with energy, an excess intake above the requirement can be tolerated without detriment to the nutriture of the individual and with no great strain on any physiological mechanism. In the dieting of individuals, a daily intake of 10 mg of calcium per kilogram of body weight would be more than enough for some and not enough for others. It is not improbable, however, that the latter group, in the course of time, could adapt themselves to the average intake of calcium. It would seem improvident and even impractical, to feed all adults a level of calcium that would cover the needs of practically all individuals, say all but 1 in 100. This would require an excess above the average of 2.33 times the standard deviation, or a total of 15.13 mg per kilogram of body weight per day, or 1.06 gm per day for a 70-kg man. To cover the needs of all but 1 in 50, the total intake of calcium should be 14.51 mg per kilogram per day, or for 70-kg man, a total intake of 1.01 gm per day.

In judging the adequacy of the calcium nutrition of a group of men, and even more so of an individual, an average requirement of calcium is of little value in itself. The variation to which it is subject, of the order of 22% in terms of the coefficient of variation is sufficient indication of its unreliability. Added to this uncertainty, is the random error to which a

coefficient of variation is subject. A coefficient of 22 obtained from a sample of 43, has a standard deviation of 2.48 (Davenport and Ekas, '36, p. 37). The probability of a considerable power of adaptation in coping with an initially inadequate calcium intake (Mitchell, '44; Kraut and Wecker, '43) must also be reckoned with. These considerations would indicate that a mere determination of the calcium intake of an individual or a community, no matter how carefully it may be accomplished, cannot in itself establish a condition of calcium under-nourishment in the adult. It may, however, afford supporting evidence in conjunction with clinical or laboratory findings indicative of a progressive osteoporosis or some other symptom that can logically be associated with an inadequate level of dietary intake.

#### SUMMARY AND CONCLUSIONS

The results of calcium metabolism studies on nineteen men, designed to afford information on the utilization of the calcium in diets containing milk products, or equally available calcium salts, to furnish about 60% of the total calcium intake, and the daily requirements of calcium, are reported in this paper. The diet periods averaged 20 days in length and a total of seventy-five such periods are considered in this report. The data appear to support the following conclusions:

1. The calcium of the experimental diets was on the average utilized to the extent of about 32% in the prevention of endogenous losses. The coefficient of variation of this average value is 23.3%.

2. The heating of milk to 160°F. for 30 minutes, the homogenization of milk, the addition of sodium alginate, citric acid or citrates in moderate amounts, the preparation of a soft curd milk by base-exchange, produced no demonstrated change in the utilization of its content of calcium.

3. The average calcium requirement of the adult human indicated by these experiments is 644 mg daily, or 9.21 mg per kilogram of body weight per day, or 343 mg per m<sup>2</sup> per day,

the coefficients of variation being, respectively, 28.1, 29.4 and 27.3.

4. The effect of body size on calcium requirements in adults, for a moderate range in size, is insignificant in comparison with other causes of variation.

5. In conjunction with other experiments of similar nature, involving a total of forty-three subjects, a good average value for the calcium requirement of adult men and women of a nutritional status representative of college students and staff members, subsisting on diets containing dairy products to furnish from one-half to two-thirds of the calcium content, is 10 mg per kilogram of body weight per day. This average is associated with a coefficient of variation of  $22 \pm 1.7$ .

6. Such an average is of value in the planning of diets, but of only subsidiary value in forming decisions as to the prevalence of calcium undernutrition in a community. Only in association with clinical or laboratory findings of symptoms indicative of a progressive draining of minerals from the bony structures, or the soft tissues of the body can a marked deficit of intake below an average requirement of calcium implicate this essential nutrient. The great variability exhibited by the human organism in its disposal of dietary calcium, involving, probably, a marked ability to adapt itself to wide ranges in calcium supply, is responsible for this situation.

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# THE EFFECT OF THE CITRATE ION ON THE CALCIUM METABOLISM OF ADULT HUMAN SUBJECTS<sup>1</sup>

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The literature on factors affecting the utilization of dietary calcium in the animal body is peculiarly conflicting, whether one considers the effect of intestinal reaction, fat, fiber, phytic acid or lactose. Citric acid, also, is no exception to this statement. Among many other organic acids, it is very completely oxidized in the body (Lanford, '42 b; Metcalf and Hathaway, '45). It is a normal constituent of bone (Dickens, '41; Class and Smith, '43). The blood citrate may constitute a physiological mechanism for the removal of calcium (Lansing and Scott, '42) and lead (Kety, '42) from the tissues and their excretion in the urine (Shorr et al., '42). Cattle and rabbits given sodium citrate intravenously, or by stomach tube, have been observed to increase their output of calcium in the urine (Marek, Wellmann and Urbányi, '42).

Dietary citrates (Hamilton and Dewar, '37), or a mixture of citric acid and citrates (Shohl, '37; Shohl and Butler, '39), have been found beneficial in the prevention or treatment of rickets, but Day ('40) claims that, in the rat at least, these beneficial effects do not develop unless there is a high calcium to phosphorus ratio in the diet, and appreciable quantities of phytin or perhaps other phosphorus-containing complexes of

<sup>1</sup> This investigation was aided by funds contributed to the University of Illinois by the Nutrition Foundation, Inc.

low biological availability: Hathaway and Meyer ('39), working with rachitic rats and large doses of citrates equivalent to over 10% of the diet, claimed special potency for potassium citrate over the sodium salt, and failed to confirm a need of a mixture of citrates with citric acid.

In normal animals and children, the effect of citrates on calcium metabolism has not been clarified by available information. Chaney and Blunt ('25) reported that in two girls, 10 and 11 years old, calcium assimilation was decidedly benefited when 600 to 700 ml of orange juice formed a part of the diet, but the general nutritional benefits in increased weight and nitrogen retention following the incorporation of orange juice in the diet suggest that other factors than citric acid were operating. Lanford ('39) has reported similar, though not so marked, effects of orange juice on calcium assimilation in the growing rat, but in later experiments ('42 a), doses of citric acid and potassium citrate equivalent to those contained in the amounts of orange juice fed, were without appreciable effect upon calcium retention. Also, Mallon and Lord ('42) observed no favorable effect of lemon juice on the calcium retention of growing rats, although lemon juice contains six times as much citric acid as orange juice (Hartmann and Hillig, '34). Watson, McGuire, Meyer and Hathaway ('45) failed to observe any demonstrable effect of either orange juice, ascorbic acid or potassium citrate (3.38 gm daily) on the calcium retentions of eight pre-school children in experiments extending over 16 weeks.

The low efficiency with which the human organism, and especially the adult organism, assimilates dietary calcium, even when milk provides two-thirds of the calcium consumed, as exemplified in the preceding paper, is a challenge to nutritional investigators to uncover some dietary expedient by which this efficiency can be raised. Even though the evidence adduced in support of a not uncommon belief that a considerable proportion of the American people are subsisting upon borderline or inadequate levels of dietary calcium is not compelling, the practical usefulness in special and emer-

gency situations of some method by which a greater proportion of food calcium than the usual 20 to 40% can be put at the disposal of the tissues, is obvious. The present report is the first attempt of the authors to find such a method and while the results are not encouraging, they seem worth reporting. Some of their implications with reference to certain phases of calcium metabolism seem clear.

#### EXPERIMENTAL METHODS

The experiments were carried out upon seventeen of the nineteen subjects described in the preceding article. The technic of feeding, collecting of excreta, and chemical analysis was the same as previously described, except as the special purposes of these studies imposed special departures. The metabolism periods were 4 days in length and, on any one dietary regime, there were usually five such consecutive periods. The basal low-calcium diets were practically the same as those used in the earlier experiments. The effect of citric acid, sodium citrate, potassium citrate and orange juice upon the assimilation of the calcium in the basal diet and in the basal diet plus milk was studied, as well as the residual effects of these supplements in periods subsequent to their discontinuance. Some information was obtained also on the utilization of the calcium in calcium citrate as compared with that of calcium gluconate. The calcium in the latter compound has been shown to be as well utilized as that of milk (Steggerda and Mitchell, '39).

#### EXPERIMENTAL RESULTS

The tests of the utilization of calcium citrate and of calcium gluconate yielded the data presented in table 1. In this table will be found the excess calcium consumed over that contained in the basal low-calcium diet, together with the improvement in calcium balance brought about by the calcium salt supplement, both expressed in milligrams per day. These data are averaged for each salt, and the utilization computed by dividing the improvement in balance by the increase in



intake and multiplying by 100. The average utilization is 30 for the citrate salt and 25 for the gluconate. The difference cannot be considered significant because of the fewness of the data and their variability.

In table 2 are assembled the data secured when potassium citrate, 9 gm daily, or a mixture of potassium citrate, 4.5 gm daily, and citric acid, 4.5 gm daily, was added to the basal low-calcium diet to see whether these supplements would alter the output of dietary calcium at this low level, or would

TABLE 1

*The utilization of the calcium in the citrate and gluconate salts.  
Average daily data.*

SUBJECT	LENGTH OF TEST	EXCESS OVER BASAL		UTILIZATION
		Intake	Balance	
	days	mg	mg	%
Calcium citrate				
Sn	20	+ 301	+ 69	
Sn	20	+ 326	+ 148	
To	20	+ 152	+ 21	
Hal	20	+ 326	+ 112	
Le	20	+ 195	+ 39	
Averages		+ 260	+ 78	30
Calcium gluconate				
Sn	20	+ 299	+ 19	
Sn	20	+ 360	+ 89	
Hal	20	+ 360	+ 103	
Le	20	+ 171	+ 90	
Averages		+ 297	+ 75	25

change the balance of calcium. However, no consistent effect of the citrate ion is revealed by these studies. For the three subjects receiving the combination, the calcium balance averaged — 104 mg daily before the addition, and — 101 mg daily after the addition. For the two subjects receiving the potassium citrate supplement alone, the balances averaged — 124 mg before dosage, and — 128 mg during the 20-day dosage period.

When either sodium citrate or citric acid was added to milk consumed in such amounts as would induce slight negative balances of calcium, or at most very slight positive balances, the utilization of calcium was not appreciably or consistently affected. The evidence for this statement will be found in table 3. The average utilization percentages for milk alone and for milk plus sodium citrate for nine subjects averaged

TABLE 2

*The effect of potassium citrate and citric acid on the utilization of calcium in the basal diet.*

Results expressed in milligrams per day per 20-day period.

SUBJECT	SUPPLEMENTS TO BASAL DIET	CALCIUM METABOLISM			
		Intake	Feces	Urine	Balance
Steg	None	216	222	106	— 112
	Potassium citrate <sup>1</sup>	209	231	60	— 82
	Potassium citrate plus citric acid <sup>2</sup>	207	249	65	— 107
Step	None	213	267	83	— 137
	Potassium citrate <sup>1</sup>	216	276	56	— 116
	Potassium citrate plus citric acid <sup>2</sup>	222	279	93	— 150
Has	None	204	190	78	— 64
	Potassium citrate plus citric acid <sup>2</sup>	211	223	92	— 104

<sup>1</sup> 9 gm daily in three equal portions at meals.

<sup>2</sup> 4.5 gm of each daily in three equal portions at meals.

the same, namely, 32. For four subjects receiving milk alone and citric acid milk in adjacent periods, the percentage utilization also averaged the same, 25.

Although the citrate ion, in the amounts used, does not appear to modify appreciably the disposal within the adult human body of moderate levels of dietary calcium, throughout a 20-day period, when the dosage is discontinued the metabolism in subsequent periods may be markedly disturbed, as the data in table 4 demonstrate. In the 40- to 56-day interim between the first and the second citrate period, the calcium metabolism of subject Ro was not greatly affected, except

for a tendency for fecal calcium to decrease and for urinary calcium to increase at a somewhat greater rate. But for subjects Te, Steg, and Be, the balances became definitely less negative or more positive, generally due to a decreasing output of calcium, following an initial increase for two of the three men. Of particular interest is the large out-pouring of

TABLE 3

*Utilization of the calcium of milk consumed alone, and, in an adjacent period, with sodium citrate or citric acid, 20-day periods except as indicated.*

SUBJECT	MILK ALONE	MILK PLUS SODIUM CITRATE <sup>1</sup>	MILK ALONE	MILK PLUS CITRIC ACID <sup>2</sup>
	%	%	%	%
Steg	22	31	..	..
Steg	8	23	..	..
Steg	..	..	17 <sup>3</sup>	19
Ve	20	36	..	..
Sc	36	26	..	..
Sc	67	35	..	..
McB	44	39	..	..
Be	0	28	..	..
Ro	29	31	..	..
Te	58	35	..	..
Ne	..	..	18	27
Cl	..	..	40	29
Fo	..	..	27 <sup>1</sup>	27
Averages	32	32	25	25

<sup>1</sup> 9 gm sodium citrate daily, given in capsules distributed evenly among the 3 meals.

<sup>2</sup> 0.27% citric acid in the milk.

<sup>3</sup> 16-day period.

calcium in the feces in the last low-calcium period for each of the four subjects, following the second citrate-feeding period. Comparing the final low-calcium period with the initial low-calcium period in this sequence of experiments for each subject, the daily fecal calcium increased 56, 89, 76, and 77%, for the four subjects in the order in which they are listed in the table. In assessing the significance of these increases, it should be remembered that the feces of successive 4-day periods were marked off with carmine, and that the data for

TABLE 4

*The effect of sodium citrate on calcium metabolism in periods subsequent to its administration.*

SUPPLEMENTS TO LOW-CALCIUM BASAL DIET	LENGTH OF PERIOD	DAILY CALCIUM METABOLISM				INDICATED UTILIZA- TION <sup>1</sup>
		Intake	Feces	Urine	Balance	
	<i>days</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>%</i>
Subject Ro						
None	16	201	232	177	— 208	
Milk + Na citrate	20	516	443	184	— 111	31
Milk	20	504	430	195	— 121	29
Milk	20	484	412	256	— 184	8
Milk	16	494	349	254	— 109	34
Milk + Na citrate	16	498	415	210	— 127	27
None	16	158	362	185	— 389	
Subject Te						
None	20	216	307	76	— 167	
Milk + Na citrate	20	498	467	98	— 67	35
Milk	20	517	444	65	+ 7	58
Milk	20	496	424	78	— 6	58
Milk + Na citrate	16	492	310	73	+ 109	100
None	6	264	581	54	— 371	
Subject Steg						
None	16	219	208	81	— 70	
Milk + Na citrate	20	518	390	129	— 1	23
Milk	20	493	445	96	— 49	8
Milk	20	517	352	101	+ 64	45
Milk	16	524	362	83	+ 79	49
Milk + Na citrate	16	494	324	86	+ 84	56
None	15	192	366	72	— 246	
Subject Be						
None	20	213	274	65	— 126	
Milk + Na citrate	20	532	466	103	— 36	28
Milk	20	535	534	128	— 127	0
Milk	20	502	408	93	+ 1	44
Milk	8	537	310	92	+ 135	81
Milk + Na citrate	16	512	396	75	+ 41	56
None	16	207	485	68	— 346	

<sup>1</sup> Computed on the basis of the first low-calcium period for each subject.

any 4-day period following a change in experimental treatment was disregarded if they were out of line with the data of the subsequent periods on the same diet. The calcium balances for all subjects were much more negative in the final low-calcium period than in the initial. The significance of these findings will be discussed later.

A daily supplement of 500 ml of orange juice (table 5) to the diets of four subjects, definitely depressed the utilization of milk calcium in subject Sw, an effect that persisted, apparently, for at least 40 days after the supplement was discontinued. In subjects Steg, Sn and To, the orange juice exerted little if any effect during the period of administration, but a more or less clear disturbing effect subsequently. This delayed effect was most pronounced with subject Sn, in whom the orange juice appeared to have induced a reduction in both fecal and urinary calcium extending over 72 days, so that the indicated utilization of milk calcium, computed on the basis of the first low-calcium period, was fictitiously high. This effect continued through a final low-calcium period. A similar reaction to orange juice, though not by any means so pronounced, was observed for subject To. The data for subject Steg extended over only one post-orange-juice period, but, such as they are, they indicated a depression of calcium utilization. For the three subjects for whom the sequence of experiments terminated in a low-calcium period, the calcium balance for the final period was much less negative, or in one case actually positive, as compared with the initial low-calcium period. This effect is just the opposite to that observed after citrate administration (table 4).

#### DISCUSSION

The experiments reported in this paper agree with most of the published evidence on normal animals and children in finding no effect of the citrate ion on calcium metabolism during a period of daily administration lasting 20 days. Also, calcium citrate does not seem to be clearly distinguished from

calcium gluconate or from milk in the utilization of its calcium by adult man.

However, after daily administration of citrates has ceased, profound effects upon calcium metabolism may ensue in subsequent periods. At approximately the same level of calcium intake, but especially on a low-calcium intake, a greatly increased output of calcium will generally occur, in feces or urine or both. Following this outpouring of calcium from the body, a reverse tendency may set in, to such an extent that the apparent utilization of calcium, computed on the basis of a low-calcium period just prior to citrate administration, may reach 80, or even 100%. However, for the entire sequence of experiments for each of the four subjects on this test (table 4), the total utilization of dietary calcium is not appreciably affected if consideration is given to the extra calcium excreted in the final, as compared with the initial, low-calcium period. If attention be restricted to the three subjects showing most distinctly the delayed effect of citrates above described, the uncorrected utilization percentages average 46, and the corrected percentages, 31.

These delayed effects of the citrate ion, extending over 40 to 60 days, can hardly relate to events occurring in the digestive tract, the contents of which are continually being removed and replaced from successive ingestions of food. Rather, they would seem to relate to metabolism and to cumulative effects of the citrate ion. The initial outpouring of calcium in the urine, but particularly in the feces, following a period of citrate administration, is in harmony with the observations of Gomori and Gulyas ('44), Lansing and Scott ('42), Shorr et al. ('42), and Marek, Wellmann and Urbányi ('42), except that these reports are concerned only with an increased output of calcium in the urine during a period of citrate dosage. The subsequent decrease in calcium output observed in our experiments, and in fact the entire picture of the delayed effect of the citrate ion on calcium metabolism, has no counter-part in anything we have found in the literature. It is hard to visualize on the basis of available informa-

tion the means by which these delayed effects are mediated. The most obvious inference is that during a period of citrate administration, citrate is stored in the body, later to be slowly released over a considerable period of time. Its effect on metabolism during the period of storage may be minimal if the storage tissues withdraw the citrate ion more rapidly from the blood stream than do the tissues subject to the metabolic influence of the ion. On discontinuance of the dosage, the storage tissues may release the ion for action elsewhere, the situation being somewhat analogous, perhaps, to the storage and release of the lead ion by the skeleton. In the case of citric acid, some 70% of the body's stores is said to be located in the skeleton (Dickens, '41), and these stores do not seem to be increased by citrate feeding (Leonards and Free, '44). These facts, if applicable to the adult human body, do not aid in explaining the delayed effects of the citrate ion on calcium metabolism.

The orange juice effect on calcium metabolism, illustrated by the data in table 5, is somewhat similar to the citrate effect for subjects Sn and To, but not for the other two men. In particular, there is no outpouring of calcium in the feces in the final low-calcium periods.

The observations reported in this paper bear upon the validity of a relatively new theory of calcium metabolism associated particularly with the names of McCance and Widdowson. Impressed by the fact that intravenous injections of calcium gluconate into normal subjects over a period of 2 weeks, in daily amounts equivalent to 186 mg of calcium, produced a rapid increase in urinary calcium but no detectable increase in fecal output of calcium, these investigators ('39) deduced the general conclusion "that the intestine does not normally excrete calcium . . . in amounts which are functions of plasma levels or metabolic requirements." This conclusion has been broadened still further into the belief that fecal calcium for all practical purposes consists entirely of calcium unabsorbed from that taken by mouth with the diet (McCance and Widdowson, '42). The fact, for example, that

TABLE 5

*The effect of orange juice on calcium metabolism in periods subsequent to its administration.*

SUPPLEMENTS TO LOW-CALCIUM BASAL DIET	LENGTH OF PERIOD	DAILY CALCIUM METABOLISM —				INDICATED UTILIZA- TION <sup>1</sup>
		Intake	Feces	Urine	Balance	
	<i>days</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>%</i>
Subject Steg						
None	20	167	231	58	— 122	
Milk	20	500	412	89	— 1	36
Milk + orange juice <sup>2</sup>	20	488	428	75	— 15	33
Milk	20	432	433	84	— 85	14
None	16	199	217	65	— 83	
Subject Sn						
None	20	261	280	182	— 201	
Milk	24	625	479	164	— 18	50
Milk + orange juice <sup>2</sup>	20	742	553	152	+ 37	49
Milk	20	657	468	129	+ 60	66
Milk	20	639	405	130	+ 104	81
Milk	12	664	523	127	+ 14	53
Chocolate milk <sup>3</sup>	20	632	430	117	+ 89	78
None	20	270	217	100	— 47	
Subject To						
None	36	192	86	210	— 104	
Milk	20	453	190	273	— 10	27
Milk + orange juice <sup>2</sup>	20	490	211	206	+ 73	43
Milk	20	418	134	186	+ 98	57
Chocolate milk <sup>3</sup>	20	396	153	155	+ 88	44
None	20	217	87	99	+ 31	
Subject Sw						
None	20	261	150	171	— 60	
Milk	24	371	197	191	— 17	40
Milk + orange juice <sup>2</sup>	20	481	309	220	— 48	5
Milk	20	401	247	277	— 123	— 45
Milk	24	386	250	262	— 126	— 52

<sup>1</sup> Computed on the basis of the first low-calcium period for each subject.

<sup>2</sup> 500 ml daily.

<sup>3</sup> In unpublished experiments on 8 adult human subjects, in cooperation with the Department of Home Economics, it was shown that chocolate in the amounts used in the preparation of chocolate milk, has no appreciable effect on the utilization of milk calcium.



an increase in protein intake of healthy adults will decrease the output of calcium in the feces and increase that in the urine has been interpreted to mean that amino acids resulting from protein digestion will increase the solubility of dietary calcium and thus its absorbability from the intestinal tract (McCance, Widdowson and Lehmann, '42). On the basis of such evidence, Hall and Lehmann ('44) have proposed the use of a calcium peptone powder to increase calcium absorption in hospital patients.

This broad interpretation of observations made under specific sets of experimental conditions is not justified and neglects entirely many observations of contrary significance made under other sets of experimental conditions. It disregards the well-established fact that the intestinal mucosa possesses more than a one-way permeability. Water and chlorides (Goldschmidt and Dayton, '19) and urea (Pendleton and West, '32) pass freely from the blood into the lumen of the alimentary tract under certain conditions. Bergeim ('26), by his method of detecting areas of absorption and areas of excretion in the tract, demonstrated clearly that, under certain conditions, calcium will pass from the blood to the interior of the intestine, an observation that has been confirmed by French and Cowgill ('37) and by Longwell in unpublished observations from this laboratory. In a more recent report, Greenberg ('45) has shown that, under his experimental conditions, 18.0 to 18.5% of an injected dose of radioactive calcium could be recovered from the feces. Possibly this effect is mediated through the bile (Greenberg and Troescher, '42).

The acidic or basic-character of the diet undoubtedly is one condition that determines to what extent absorbed calcium will be excreted through the intestinal walls and be voided with the feces. The reports of Zucker ('21) and of Bogert and Kirkpatrick ('22) show that acidic diets divert absorbed calcium to the urine, while alkaline diets divert absorbed calcium to the feces, often with little disturbance of the balance of calcium in the body. Farquharson, Salter and Aub

('31) showed that the tendency of a high-protein diet to divert absorbed calcium to the urine because of its acidic character could be obviated by simultaneous ingestion of  $\text{NaHCO}_3$ , both with a low-calcium and a moderate-calcium diet. In animals, such as the rabbit or the dairy cow, whose rations are habitually alkaline after oxidation, the urine contains very little calcium (Forbes et al., '22), but when given acid, the urinary output of calcium may increase enormously, while the fecal output decreases proportionately (Granström, '08).

The literature cited is only a sample of the evidence that could be marshalled against the contention that fecal calcium is unabsorbed calcium. It is true that much of this evidence is not conclusive, because it is subject to two interpretations, either the obvious one given to it in the above discussion, or the less probable one that the calcium in all potentially alkaline foods is less absorbable than that in potentially acid foods; or that acid ingested with the food always accelerates calcium absorption and that alkali depresses it. Some doubt has been cast upon the latter theory by Jones ('42).

The data contained in table 4, however, are unequivocal in their significance. Here, in four adult human subjects, the prior feeding of sodium citrate has, in the final low-calcium period, increased the fecal calcium output over that in the initial low-calcium period, by 56, 89, 76 and 77%, respectively, on generally lower intakes of calcium. In this final period, the fecal calcium exceeded the intake by 129, 120, 91, and 134%. Whether this represents a diversion of calcium from urine to feces by the alkalinity produced in the body on the oxidation of the acid radicle of sodium citrate, or some more specific effect of the citrate ion, cannot be decided. Unfortunately, the pH of the urines was not followed in these series of experiments. But regardless of the explanation, the data show indubitably that calcium is passing in large amounts from the blood stream to the lumen of the gastro-intestinal tract and that under these conditions, fecal calcium is obviously much more than unabsorbed food calcium.

## CONCLUSIONS

1. Calcium citrate is approximately as well utilized by the adult human organism as is calcium gluconate.

2. Neither potassium citrate, nor a mixture of potassium citrate and citric acid, appreciably modifies the utilization of the calcium in a basal low-calcium diet.

3. The utilization in the adult organism of the calcium of milk is not appreciably or consistently modified by the simultaneous ingestion of sodium citrate, citric acid or orange juice in considerable amounts.

4. Sodium citrate and orange juice may induce profound disturbances in the calcium metabolism of adult men subsequent to a 20-day period of dosage, characterized generally by a phase of increased retention of calcium, followed, or preceded, or both, by a period of increased excretion. These changes are often beyond the limits of variation due to uncontrolled experimental factors and seem clearly traceable to the prior administration of the citrate ion.

5. Following citrate administration, the calcium in the feces of subjects subsisting on a low-calcium diet may amount to over twice the calcium intake. This is merely another instance, of many cited, in which fecal calcium contains much more than unabsorbed dietary calcium.

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# DENTAL CARIES IN THE COTTON RAT

## VI. THE EFFECT OF THE AMOUNT OF PROTEIN, FAT AND CARBOHYDRATE IN THE DIET ON THE INCIDENCE AND EXTENT OF CARIOUS LESIONS <sup>1</sup>

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A high incidence and extent of carious lesions occurred in the molars of cotton rats fed diets high in sucrose or other soluble carbohydrates (Shaw et al., '44, '44 a; Schweigert et al., '45, '45 a; Shaw et al., '45). A low incidence and extent were noted when sufficient fluorine was added to the sucrose control ration, or when dextrin and stock rations were fed. This work has been extended to a study of the effect of the amount of fat, protein and carbohydrate in the ration on the incidence and extent of the lesions. The effect of feeding milk diets has also been studied. The results of these experiments are reported in this paper.

### EXPERIMENTAL

The procedures described by Schweigert et al. ('45) have been followed in weaning the animals, dividing litter mates between groups on experiment, and calculating the growth

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rates. The method of evaluating the incidence and extent of the carious lesions after the 14-week experimental period has been reported by Shaw et al. ('44, '44 a).

The composition of the sucrose control ration 802, medium and high fat diets (821 and 814), high protein-medium fat diet (822) and high protein ration (813) is given in table 1. Four per cent of 1:20 liver extract was added to all diets at the expense of the entire ration and adequate quantities of the B vitamins were provided (McIntire, Schweigert and Elvehjem, '44). Each rat received 1 drop of halibut liver oil per week. In rations 814, 821, and 822, the lard was added isocalorically at the expense of the sucrose, thus maintaining the same mineral-vitamin-energy relation.

Whole liquid milk (fortified with iron, copper and manganese<sup>3</sup>) was fed to cotton rats as the sole source of food and water. 1:20 liver extract has been shown to increase the growth rate when added to purified rations (McIntire et al., '44; Schweigert et al., '45) without altering the incidence and extent of carious lesions (Shaw et al., '44). Therefore, for some groups, liver extract was homogenized into the milk at a level equivalent to 4% of the milk solids (0.5 gm of 1:20 liver extract per 100 ml of milk). A diet approximating the composition of milk solids (Rodgers, '35) was fed to compare the incidence and extent of tooth decay with those observed when liquid milk was fed. The composition of this ration (815) is given in table 1. Adequate quantities of the B vitamins (McIntire et al., '44) were provided and 4% of 1:20 liver extract was added at the expense of the entire ration.

## RESULTS

The growth rates of cotton rats for the first 6 weeks and for 14 weeks on experiment and the incidence and extent of tooth decay are shown in table 2. A difference in the susceptibility to tooth decay between offspring from different parent stock has been noted in earlier work (Schweigert et al., '45 a);

<sup>3</sup> One mg of iron and 0.1 mg of copper and manganese were supplied daily to each animal.

therefore, the incidence and extent of dental caries observed for each experimental group and also for the litter mate controls fed the sucrose basal ration are presented in table 2.

The diets containing lard supported good growth, equal to that observed when the basal ration was fed. The growth rates when high protein diets were fed, also were similar to those observed for rats receiving the sucrose control ration. The growth rate observed when the rats were fed only mineralized whole milk was inferior to that observed when

TABLE 1

*Composition of rations.*

*(The amount of each constituent is expressed in terms of parts of the ration.)*

RATION	802 (SUCROSE BASAL)	918 (HIGH PROTEIN)	814 (HIGH FAT)	815 (MILK SOLIDS)	821 (MEDIUM FAT)	822 (HIGH PROTEIN- MEDIUM FAT)
<i>Constituent</i>						
Sucrose	67	41	22		44.5	18.5
Salts IV	4	4	4	5.4	4	4
Casein	24	50	24	27.3	24	50
Corn Oil	5	5	5		5	5
Lard			20		10	10
Lactose				39		
Butterfat				28.3		
Percentage of fat in the protein, carbo- hydrate and fat portion of the diet	5.2	5.2	35	30	18	18

the control ration was fed. The addition of 4% of 1:20 liver extract to the milk increased the growth rate approximately 2 gm per week for the first 6 weeks; however, the growth rate was still not as rapid as when the control ration was fed. The milk solids ration (815) supported better growth than whole milk but was inferior to the control diet. Apparently the cotton rat cannot tolerate high levels of lactose in the diet since the growth rate was retarded and a high mortality occurred when such rations were fed (table 2 and unpublished



TABLE 2  
*Effect of the level of fat and protein and milk diets on the incidence and extent of carious lesions.*

RATON	NUMBER OF ANIMALS	GM GAINED/WEEK		AVE. INCIDENCE OF CARIOUS LESIONS	AVE. EXTENT OF CARIOUS LESIONS
		6 weeks	14 weeks		
802 + 4% 1:20 L.E.	3	9.3	7.1	33	113 +
814 (high fat) + 4% 1:20 L.E.	4	10.1	7.1	6	7 +
802 + 4% 1:20 L.E.	3	10.0	7.0	31	91 +
814 (high fat) + 4% 1:20 L.E.	4	9.0	6.1	3	4 +
821 (medium fat) + 4% 1:20 L.E.	4	9.2	6.9	13	24 +
802 + 4% 1:20 L.E.	4	10.5	7.6	28	78 +
821 (medium fat) + 4% 1:20 L.E.	4	11.1	8.1	13	29 +
802 + 4% 1:20 L.E.	3	9.3	7.1	33	113 +
813 (high protein) + 4% 1:20 L.E.	2	7.5	8.9	21	37 +
802 + 4% 1:20 L.E.	4	10.3	8.2	24	69 +
813 (high protein) + 4% 1:20 L.E.	5	8.8	6.0	11	24 +
822 (high protein-medium fat) + 4% 1:20 L.E.	5	9.7	7.1	1	1 +
802 + 4% 1:20 L.E.	3	10.6	6.1	23	67 +
Mineralized whole milk	5	5.0	4.5	0	0
Mineralized whole milk + 0.5 gm 1:20 L.E./100 ml	4	6.9	5.0	0	0
802 + 4% 1:20 L.E.	3	10.2	6.8	32	104 +
Mineralized whole milk + 0.5 gm 1:20 L.E./100 ml	3	8.3	5.3	0	0
802 + 4% 1:20 L.E.	3	11.1	8.5	20	58 +
815 (milk solids) + 4% 1:20 L.E.	3	8.1	6.6	5	7 +
802 + 4% 1:20 L.E.	6	8.9	7.2	32	108 +
815 (milk solids) + 4% 1:20 L.E.	3 <sup>1</sup>	6.4	6.3	2	6 +

<sup>1</sup> Six animals were started in this group, but 3 failed to survive the 14-week experiment.

data). This fact has limited the amount of work that could be done with high lactose diets.

The ingestion of diets containing additional fat (814 and 821) resulted in a marked reduction in the caries occurrence. When 20 parts of lard were substituted at the expense of the sucrose, the average incidence and extent were 6 and 7 + and 3 and 4 + for the two groups, respectively. These figures are in contrast to the average incidence and extent of 33 and 113 + and 31 and 91 +, respectively, which were observed when the sucrose ration was fed to their litter mate controls. An intermediate caries incidence and extent were noted, 13 and 24 + and 13 and 29 +, respectively, when 10 parts of lard were substituted for sucrose in ration 821. Some reduction in the carious occurrence was noted when a 50% casein diet was fed (813), but when ration 822 was fed (10 parts of lard and 50 parts of casein) a further reduction in tooth decay was observed. The average incidence and extent of the lesions for the two groups when ration 813 was fed were 21 and 37 + and 11 and 24 +, respectively (table 2). When ration 822 was ingested an incidence of 1 and an extent of 1 + were obtained.

No cavities were observed in twelve animals that had been fed mineralized whole milk or mineralized whole milk plus 1:20 liver extract (table 2). In previous work when dextrin or stock rations were fed, a low incidence and extent were observed, but the protection was not as complete as when milk was fed. A low incidence and extent of tooth decay were noted when the milk solids ration (815) was fed. These results were comparable to those observed when the high fat ration 814 was fed.

#### DISCUSSION

These data show some of the interrelationships between the level and kind of carbohydrate, the amount of protein and fat in the diet and the incidence and extent of carious lesions in the cotton rat.

It has been found that partial caries protection was afforded when 50 parts of dextrin and 17 parts of sucrose were fed

in place of 67 parts of sucrose (Schweigert et al., '45). When dextrin was fed as the sole carbohydrate, a very low incidence and extent of lesions were noted. However, the ingestion of ration 821 (44.5 parts of sucrose and 10 parts of lard) resulted in a greater protection than when the above dextrin-sucrose ration was fed. In fact, when ration 814 was fed (20 parts of lard and 22 parts of sucrose) almost complete protection was observed, in spite of the fact that this ration contained more sucrose than the dextrin-sucrose diet (1.42 times as much on a percentage basis). Therefore, the effect of fat appeared to be more pronounced than that of dextrin in reducing the caries incidence.

The amount of tooth decay observed when ration 813 was fed (50 parts casein and 41 parts sucrose) was much lower than when the sucrose control ration was fed. The added protein in this ration (26 parts) appeared to produce about the same reduction in the number and extent of cavities as the substitution of 10 parts of lard to the diet (ration 821) and a greater reduction than the substitution of 50 parts of the sucrose with dextrin. The additive effect of 50 parts of casein and 10 parts of lard in the diet (18.5 parts of sucrose) was observed when the results were compared with those on high protein or 10 parts of lard in the diet. The protection was comparable to that observed when 20 parts of lard and 22 parts of sucrose were fed in ration 814.

These results help to explain why milk afforded complete protection against dental caries in the cotton rat. Milk on a solids basis is a high fat ration and the ingestion of fat has been shown to markedly reduce the caries occurrence. The presence of lactose as the sole carbohydrate in milk and a slightly higher level of protein may also have contributed to the low caries incidence observed.

Becks and coworkers ('44) have shown that a marked reduction in the frequency of dental caries in human subjects occurred when the intake of refined carbohydrates was reduced by replacement of the carbohydrates with meat, eggs, milk and milk products. The latter foods would not only

supply a small amount of carbohydrate, but would increase the fat and good quality protein levels of the diet. Jay et al. ('36) and Koehne et al. ('34, '34 a) have noted that the incidence of caries in humans was increased when sugar or "sweet foods" were ingested in appreciable amounts. Read and Knowles ('38) observed that in a caries-free group of children, the intake of fat and protein was good while in a caries-susceptible group the protein and fat intakes were deficient with the amount of carbohydrate consumed consequently increased. Boyd ('44) observed that the caries experience in 2 groups of children was comparably reduced even though the fat content of the diets differed by almost 100%. Therefore, he attributed the low number of cavities observed to the high nutritive value of the 2 diets and not to the low carbohydrate, high fat composition. The ratio of protein : carbohydrate : fat in the high and low fat diets was 7 : 9 : 21 and 7 : 15 : 11, and the percentage of fat was 57 and 33, respectively. In the present work fat comprised 35 and 18% of the protein, carbohydrate and fat portion of the high and medium fat diets, respectively (table 1). Therefore, the fat content of Boyd's lower fat diet was roughly equivalent to the fat content of the high fat diet used in these experiments. The amount of fat in his diets may be sufficiently high so that no difference in caries activity due to the fat content of the 2 diets could be noted. In earlier work Boyd et al. ('29) and Drain and Boyd ('30) offered additional evidence in support of the value of diets of high nutritive quality for reducing the caries experience in humans. Special diets were devised for four cases with celiac disease, with protein and dextrose supplying the energy (the latter as much as 60% of the total calories over a period of months) and with special emphasis on avoidance of fat and starch. No new caries were noted and all active caries were arrested by this dietary regimen in less than 10 weeks of dietary supervision.

The data obtained with the cotton rat indicate that high levels of fat and protein do not reduce the caries incidence by merely reducing the sucrose content of the diet, since con-

siderably higher amounts of sucrose are present in these diets than in dextrin-sucrose diets where a high caries incidence occurred.

Fat and protein are probably not as favorable for rapid fermentation by the acid-producing organisms associated with tooth decay processes. The dominating types of organisms in the oral cavity may be quite different when higher proportions of fat and protein are fed at the expense of sucrose in the ration. The effect of fat may be "physical" in that it forms a protective coating over the areas of the teeth most susceptible to invasion by the bacteria present in the mouth. A study of the effect of other dietary proteins and fats, in combination with several carbohydrates is needed to determine the complete interrelationship of the effects of the amount and kind of carbohydrate, protein and fat on the incidence and extent of dental caries in the cotton rat.

#### SUMMARY

1. The isocaloric substitution of 10 or 20 parts of lard for sucrose in a purified ration reduced the incidence and extent of carious lesions in the cotton rat in proportion to the amount of lard added.
2. When the casein content of the diet was increased from 24 to 50% at the expense of sucrose, some reduction in caries occurrence was observed.
3. When 50 parts of casein and 10 parts of lard were fed, the protective effect was additive. The number of cavities observed was comparable to that observed when the ration contained 20 parts of lard.
4. No carious lesions were noted when mineralized whole milk diets were fed. The incidence and extent of tooth decay were low when a ration approximating milk solids in composition was fed.

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# THE INFLUENCE OF AUTOCLAVING SOYBEAN OIL MEAL ON THE AVAILABILITY OF CYSTINE AND METHIONINE FOR THE CHICK <sup>1</sup>

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It has been recognized for several years that cooking soybeans increases their protein nutritive value (Osborne and Mendel, '17; Hayward, Steenbock, and Bohstedt, '36; Hayward, '37). Hayward and Hafner ('41) and Almquist, Mecchi, Kratzer and Grau ('42) have demonstrated that the addition of dl-methionine to a diet containing raw soybean protein improved growth to a greater extent than when added to a diet containing the cooked soybean protein. The difference in the effectiveness of methionine observed by these investigators suggests that cooking the soybean oil meal increased the availability of cystine and methionine for the chick. Heat-treated soybean oil meal was shown by Almquist et al. ('42) to be slightly deficient in methionine for the chick when fed as the sole source of protein.

A wide variability in the nutritive value of the proteins of different commercial soybean oil meals was found by Draper and Evans ('44). The data obtained by Evans and St. John ('45) indicate that the differences in the availability of the proteins of the meals were caused by differences in extent of denaturation of the proteins by heat treatment. Hayward, Halpin, Holmes, Bohstedt and Hart ('37) found that heating at 140°C.

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for  $2\frac{1}{2}$  minutes or at  $105^{\circ}\text{C}$ . for 90 minutes produced a soybean oil meal with a higher protein nutritive value than did heating at  $105^{\circ}\text{C}$ . for 2 minutes or  $82^{\circ}\text{C}$ . for 90 minutes. Parsons ('43) obtained better growth of rats with a soybean oil meal autoclaved at  $110^{\circ}\text{C}$ . for 30 minutes than with one autoclaved at  $123^{\circ}\text{C}$ . for 30 minutes. Bird and Burkhardt ('43) obtained better chick growth with a soybean oil meal autoclaved for  $2\frac{1}{2}$  minutes at  $128^{\circ}\text{C}$ . than with one autoclaved for longer or shorter times at that temperature.

Marvel, Carrick, Roberts and Hauge ('44, '45) concluded that soybean oil meals were deficient in choline as well as methionine for growing chicks and that choline and methionine acted interchangeably to supplement the diet they used.

The purpose of the present investigation was to determine the influence of autoclaving soybean oil meal at different temperatures on the growth of chicks and on the availability of the cystine and methionine of soybean oil meal for chicks.

#### EXPERIMENTAL

Solvent-extracted soybean flakes<sup>2</sup> were used in this study. These flakes were ground in a hammer mill and well mixed. The required amount of the raw soybean oil meal for each diet was weighed into enameled pans. Each sample was spread in the bottom of the pan to a depth of about 1 inch. The meals were autoclaved for 30 minutes at steam pressures required to give temperatures of  $100^{\circ}\text{C}$ .,  $110^{\circ}\text{C}$ .,  $120^{\circ}\text{C}$ ., and  $130^{\circ}\text{C}$ . One sample was autoclaved at  $130^{\circ}\text{C}$ . for 60 minutes.

Each of the diets fed had the following composition: cere-lose, 53.0 gm; soybean oil meal, 29.0 gm; dried brewer's yeast, 5.0 gm; gelatin, 5.0 gm; mineral mixture,<sup>3</sup> 5.0 gm; soybean oil, 2.5 gm; fortified fish oil, 0.5 gm; tocopherol concentrate, 10.0 mg; p-aminobenzoic acid,<sup>4</sup> 10.0 mg; nicotinic acid, 3.0 mg;

<sup>2</sup> Supplied by Spencer — Kellog and Sons, Inc., Buffalo, N. Y.

<sup>3</sup> The composition of the mineral mixture was: oyster shell, 1570 gm;  $\text{CaHPO}_4$  ·  $2\text{H}_2\text{O}$ , 1740 gm;  $\text{K}_2\text{HPO}_4$ , 840 gm;  $\text{NaCl}$ , 600 gm;  $\text{MgSO}_4$  ·  $7\text{H}_2\text{O}$ , 500 gm;  $\text{Fe}_2(\text{SO}_4)_3$  ·  $\text{XH}_2\text{O}$ , 55 gm;  $\text{MnSO}_4$  ·  $4\text{H}_2\text{O}$ , 29 gm;  $\text{KI}$ , 3.3 gm;  $\text{CuSO}_4$  ·  $5\text{H}_2\text{O}$ , 1.5 gm;  $\text{ZnCl}_2$ , 1.0 gm; and  $\text{CoCl}_2$  ·  $6\text{H}_2\text{O}$ , 0.2 gm.

<sup>4</sup> The crystalline vitamins were supplied by Hoffman-LaRoche, Inc., Nutley, N. J.

2-methyl-1, 4-naphthoquinone, 1.0 mg; riboflavin, 1.0 mg; calcium pantothenate, 1.0 mg; thiamine, 0.5 mg; and pyridoxine, 0.5 mg. The diets contained 21% protein. The only differences in the diets fed the different groups were in the heat treatment of the soybean oil meal and the additions of choline and methionine<sup>5</sup> to some of the diets. Descriptions of the heat treatment and per cent choline and methionine added to each diet are given in table 1.

Seventeen groups of fifteen New Hampshire chicks each were used in the experiment and were kept in electrically heated battery brooders with wire floors. The chicks were weighed individually at weekly intervals, and at the same time feed consumption was recorded. The duration of the experiment was 4 weeks. In order to determine the efficiency of utilization of cystine and methionine the droppings were collected on glass plates during the third week of the experiment. A careful record of gain in body weight and feed consumption was also kept for the third week. The droppings were air-dried, weighed, ground in a Wiley Mill, and sampled for chemical analysis.

Total sulfur was determined on the diets fed and on the droppings by the method described by Evans and St. John ('44). Inorganic and organic sulfur were determined as described by Evans and Greaves ('37), and cystine and methionine by the differential oxidation procedure described by Evans ('45). The cystine and methionine method is based on the principle that cystine is oxidized to sulfate by concentrated  $\text{HNO}_3$ , but methionine is not. Methionine is calculated from the total sulfur minus the sulfur oxidized to sulfate by  $\text{HNO}_3$ . Cystine is calculated from the sulfur oxidized to sulfate by  $\text{HNO}_3$  minus inorganic sulfur. Cystine sulfur plus methionine sulfur thus equals organic sulfur. The retention of organic sulfur, cystine, and methionine by the growing chick was calculated from the results of the chemical analysis, and the amounts of feed consumed and droppings excreted.

<sup>5</sup> The methionine was supplied by Dr. L. C. Norris, Cornell University, Ithaca, N. Y.

TABLE 1  
*The influence of heat treatment and additions of choline and methionine on the nutritive value of soybean proteins.*

GROUP NO. <sup>1</sup>	HEAT TREATMENT OF SOYBEAN MEAL		ADDITIONS TO DIET		WEIGHT AT 4 WEEKS (Average)	GAIN IN WEIGHT AT 4 WEEKS (Average)		PROTEIN EFFICIENCY (GAIN/PROTEIN)
	Time	Temp. °C	Choline %	Methionine %	gm	gm	gm	
1	None				98 ± 19	58	1.14	
2	30	110			192 ± 36	154	1.66	
3	30	130			146 ± 33	106	1.43	
4	None		0.2		114 ± 32	76	1.24	
5	30	100	0.2		212 ± 33	174	1.81	
6	30	110	0.2		194 ± 33	156	1.76	
7	30	120	0.2		202 ± 44	162	1.66	
8	30	130	0.2		159 ± 42	120	1.57	
9	60	130	0.2		108 ± 22	69	1.09	
10	None			0.2	188 ± 41	149	1.71	
11	30	110		0.2	266 ± 40	226	2.09	
12	30	130		0.2	253 ± 42	215	1.90	
13	None		0.2	0.2	176 ± 22	137	1.90	
14	30	110	0.2	0.2	298 ± 49	260	2.19	
15	30	130	0.2	0.2	261 ± 41	223	1.95	
16 <sup>2</sup>	Commercial meal				183 ± 49	145	1.71	
17	Practical <sup>3</sup> diet				254 ± 26	214	1.81	

<sup>1</sup> Each group contained 15 chicks.

<sup>2</sup> The soybean oil meal used in this diet was commercially prepared.

<sup>3</sup> The practical diet was a commercial type diet such as would usually be fed growing chicks.

## RESULTS

Total gain in body weight and gain per gram of protein consumed were used as criteria of the nutritive value of the proteins of the soybean oil meal and are presented in table 1. The raw soybean oil meal with no dietary supplement supported very poor growth in chicks. Autoclaving at 110°C. for 30 minutes increased the protein nutritive value of the soybean oil meal for chicks fed the basal diet alone or with added methionine, or methionine plus choline. Autoclaving at 130°C. increased the protein nutritive value, but not to as great an extent as autoclaving at 110°C.

When 0.2% choline was added to the basal diet, the soybean oil meals which had been autoclaved at 100, 110, or 120°C. gave better growth than the raw soybean oil meal or the soybean oil meals that were autoclaved at 130°C. for 30 or 60 minutes. The detrimental effect of overheating soybean oil meal on growth of chicks is shown in the group receiving the meal autoclaved for 60 minutes at 130°C., which had poorer total gain and gain per gram of feed consumed than the group receiving the raw meal.

The methionine content of the unsupplemented diet was 0.35% and the cystine plus methionine content was 0.63%. The addition of 0.2% dl-methionine to the diet raised the methionine content to 0.55% and the cystine plus methionine to 0.83%. The addition of 0.2% methionine in all cases greatly increased the body weights and the protein efficiency of chicks at 4 weeks over the groups receiving no added methionine. This level of methionine increased the total gain and gain per gram of protein consumed of chicks receiving raw soybean oil meal so that they equaled the values for the best heat-treated meal when unsupplemented with methionine. The addition of methionine to the soybean oil meal autoclaved at 130°C. for 30 minutes also increased its protein nutritive value so that it equaled that of the unsupplemented soybean oil meal autoclaved for 30 minutes at 110°C. The growth responses obtained by autoclaving the soybean oil meal at 110°C. and by supplementing the raw meal with 0.2% methionine

were approximately equal. The growth responses obtained separately by autoclaving at 110°C. and by supplementing with methionine were approximately additive when these treatments were combined.

The availability of the cystine and methionine in the different diets was determined by a sulfur balance study. The organic sulfur balance data are presented in table 2. Retention of organic sulfur is presented as grams retained and as per cent retained of the organic sulfur consumed, both for the total organic sulfur intake and the intake of organic sulfur from the basal diet. In calculating this latter value, it was assumed that all of the added dl-methionine was retained. This assumption appears logical since dl-methionine is fully utilized by the chick (Grau and Almquist, '43) and since no methionine appeared to be oxidized to sulfate. Further support of the assumption is that if such were not the case, the addition of methionine would appear to very greatly increase the methionine retention from the basal diet.

Chicks receiving soybean oil meal autoclaved at 100, 110, or 120°C. had a larger organic sulfur retention than chicks receiving the raw meal, whether supplemented with choline or methionine or receiving no supplements. Chicks receiving soybean oil meal autoclaved at 130°C. for 30 or 60 minutes retained a lower percentage of the organic sulfur than the chicks receiving soybean oil meal autoclaved at 100° or 110°C. for 30 minutes except that groups 12 and 15 (130°C.), which received added methionine, retained as high a percentage of the organic sulfur as groups 11 and 14 (110°C.).

The addition of 0.2% choline slightly increased the retention of organic sulfur in the group receiving raw soybean oil meal. The addition of 0.2% dl-methionine to the diets containing no added choline increased the percentage retention of the organic sulfur from the basal diets. Addition of choline plus methionine did not increase the retention of the organic sulfur of the diet.

There was a highly significant coefficient of correlation of +0.813 between the grams gain by chicks per gram of pro-

TABLE 2  
*Organic and inorganic sulfur balance of growing chick for 1-week period.*

GROUP NO.	HEAT TREATMENT OF SOYBEAN MEAL		ADDITIONS TO DIET		ORGANIC S INTAKE FROM ADDED METH. IONINE		ORGANIC S FROM BASAL DIET		TOTAL ORGANIC S RETAINED		ORGANIC S FROM BASAL DIET RETAINED <sup>1</sup>		INORGANIC SULFUR	
	Time	Temp.	Choline	Methio- nine	%	gm	gm	gm	%	gm	gm	%	gm	gm
	min	°C.											Intake	Balance
1	None					1.28	0.85	0.43	34	0.43	34	0.43	0.90	+ 0.16
2	30	110				2.89	1.38	1.51	52	1.51	52	1.51	2.03	+ 0.44
3	30	130				2.04	1.20	0.84	41	0.84	41	0.84	1.43	+ 0.19
4	None		0.2			1.61	0.83	0.78	48	0.78	48	0.78	1.13	+ 0.22
5	30	100	0.2			3.22	1.32	1.90	59	1.90	59	1.90	2.27	+ 0.51
6	30	110	0.2			2.77	1.29	1.48	53	1.48	53	1.48	1.95	+ 0.51
7	30	120	0.2			3.07	1.31	1.76	57	1.76	57	1.76	2.16	+ 0.62
8	30	130	0.2			2.22	1.23	0.99	45	0.99	45	0.99	1.56	+ 0.39
9	60	130	0.2			1.55	1.00	0.55	35	0.55	35	0.55	1.09	+ 0.29
10	None		0.2	0.2		2.52	0.71	1.81	57	1.81	57	1.81	1.78	+ 0.39
11	30	110		0.2		3.66	1.04	3.21	68	2.17	59	2.58	1.87	+ 0.71
12	30	130		0.2		3.60	1.02	3.19	69	2.17	60	2.54	1.39	+ 1.15
13	None		0.2	0.2		2.58	0.73	1.68	51	0.94	36	1.82	1.43	+ 0.39
14	30	110	0.2	0.2		3.50	0.99	2.71	60	1.72	49	2.46	1.89	+ 0.57
15	30	130	0.2	0.2		3.74	1.06	2.83	59	1.77	47	2.63	1.96	+ 0.67
16	Commercial meal					2.78	1.33	1.45	52	1.45	52	1.45	1.57	+ 0.37
17	Practical diet					6.18	2.84	3.34	54	3.34	54	3.34	1.24	+ 0.00

<sup>1</sup> This assumes that all added dl-methionine is utilized and retained.

tein consumed (table 1) and the per cent of organic sulfur retained of that ingested by the chicks (table 2) for the seventeen diets. This highly significant correlation indicates that the availability of the organic sulfur limited the extent to which the protein was utilized by the growing chick under the conditions of this experiment.

The results of the cystine and methionine balance studies are presented in table 3. The cystine sulfur was very poorly retained. The soybean oil meals heated at 100°, 110°, or 120°C. gave a better retention of cystine than the raw meals or those heated at 130°C., whether supplemented with choline or receiving no supplements, except for groups 12 and 15. Retention of methionine sulfur was improved by heating the meals at 100°, 110°, or 120°C. Heating at 130°C. increased methionine retention but not as much as heating at 110°C. Addition of methionine to the diet containing no added choline increased methionine retention, but when added to the diet containing added choline no increase in methionine retention occurred.

There appeared to be some retention of inorganic sulfur, since every group had a larger intake of inorganic sulfur than was excreted (table 2).

#### DISCUSSION

According to Grau and Almquist ('43) the methionine requirement for growing chicks is 0.5 to 0.6% of the diet and the cystine plus methionine requirement is 1.0 to 1.1%. All diets used in this experiment except the practical diet were deficient in methionine plus cystine according to these standards. The diets to which no supplemental methionine was added were deficient in methionine. With the exception of the raw meal, the growth and protein efficiency were better on the diets to which methionine was added than on the practical diet which contained 0.46% methionine and 1.03% cystine plus methionine. It is not known whether the addition of methionine to the practical diet or the addition of cystine to the diets containing added methionine would have further increased growth and protein efficiency. The very good growth

TABLE 3  
*Cystine and methionine balance of growing chicks for 1-week period.*

GROUP NO.	HEAT TREATMENT OF SOYBEAN MEAL		ADDITION <sup>1</sup> TO DIET		METHIO- NINE S INTAKE FROM BASAL DIET		METHIO- NINE S INTAKE FROM ADDED METHIO- NINE		METHIONINE S EXCRETED		METHIO- NINE S RETAINED		METHIONINE S FROM BASAL DIET <sup>1</sup>		CYSTINE S INTAKE FROM BASAL DIET		CYSTINE S EXCRETED		CYSTINE S RETAINED	
	Time	Temp.	Chol- ine	Methio- nine	%	gm	%	gm	gm	gm	%	%	gm	gm	gm	gm	gm	gm	gm	%
1	None					0.64			0.27	0.37	58	58	0.37	58	0.64	0.57	0.07	11		
2	30	110				1.44			0.37	1.07	74	74	1.07	74	1.44	1.01	0.43	30		
3	30	130				1.02			0.30	0.72	71	71	0.72	71	1.02	0.89	0.13	13		
4	None		0.2			0.81			0.23	0.58	72	72	0.58	72	0.81	0.60	0.21	26		
5	30	100	0.2			1.61			0.24	1.37	85	85	1.37	85	1.61	1.07	0.54	34		
6	30	110	0.2			1.38			0.30	1.08	78	78	1.08	78	1.38	0.99	0.39	28		
7	30	120	0.2			1.54			0.28	1.26	82	82	1.26	82	1.54	1.02	0.52	34		
8	30	130	0.2			1.11			0.30	0.81	73	73	0.81	73	1.11	0.93	0.18	16		
9	60	130	0.2			0.78			0.31	0.47	60	60	0.47	60	0.78	0.69	0.09	12		
10	None			0.2		1.26		0.71	0.36	1.61	82	82	0.90	71	1.26	1.04	0.22	17		
11	30	110		0.2		1.83		1.04	0.28	2.59	90	90	1.55	85	1.83	1.20	0.63	34		
12	30	130		0.2		1.80		1.02	0.37	2.45	87	87	1.43	79	1.80	1.06	0.74	41		
13	None		0.2	0.2		1.29		0.73	0.54	1.48	73	73	0.75	58	1.29	1.10	0.19	15		
14	30	110	0.2	0.2		1.75		0.99	0.43	2.31	84	84	1.32	75	1.75	1.35	0.40	23		
15	30	130	0.2	0.2		1.87		1.06	0.51	2.42	83	83	1.36	73	1.87	1.46	0.41	22		
16	Commercial meal					1.39			0.40	0.99	71	71	0.99	71	1.39	0.92	0.47	34		
17	Practical diet					2.43			0.83	1.60	66	66	1.60	66	2.43	2.01	0.42	46		

<sup>1</sup> This assumes that all added dl-methionine is utilized and retained.



and protein efficiency of the chicks receiving the soybean oil meal that had been autoclaved at 110°C. and 0.2% supplemental methionine suggests the possibility of less than 1.0% cystine plus methionine being required by the chick when more than 0.5% is present as methionine. Methionine was much better utilized than cystine.

Choline and methionine did not exert an interchangeable supplementary action on the diet used. This does not agree with the findings of Marvel, Carrick, Roberts, and Hauge ('44) using a corn and soybean oil meal diet. Choline may have supplemented all of the diets to a slight extent, but methionine had a much greater supplementary action.

The protein nutritive value of raw soybean oil meal was greatly increased by autoclaving for 30 minutes at 100°, 110°, or 120°C., whether or not the diet contained added choline or methionine. Since the addition of 0.2% methionine to the diet containing raw soybean oil meal increased the protein nutritive value almost as much as did the best heat treatment, it would appear that autoclaving increased the availability of the cystine and methionine, as was believed by Hayward and Hafner ('41) and Almquist, Mecchi, Kratzer, and Grau ('42). This is further substantiated by the greater retention of cystine and methionine from the diets containing the moderately heated soybean oil meals (100–120°C.). Further heat treatment decreased the protein nutritive value of the soybean oil meal. This appeared to be caused by a decreased availability of the cystine and the methionine since the addition of methionine to the overheated meals increased growth and gain per gram of protein consumed to equal those of the soybean oil meal autoclaved for 30 minutes at 110°C. and since there was a poor retention of the dietary cystine and methionine on the diets containing the overheated soybean oil meals.

It is probable that the higher autoclaving temperature did not exert the harmful effect on the soybean oil meal simply by decreasing the availability of the methionine and cystine, but since the diet was deficient in methionine this amino acid was the limiting one in all cases. The decrease in protein

nutritive value was thus due to lowered methionine availability even though the availability of all amino acids may have been decreased. Evans and St. John ('45) showed a progressive denaturation of soybean proteins with increased heat treatment of the soybean oil meal. According to data presented in the present paper this denaturation was accompanied by a decreased availability of methionine. The decreased methionine availability may have been caused by a lowered availability of some of the denatured proteins. It appears that the decreased availability was not caused by a decreased digestibility according to data obtained by Johnson, Parsons, and Steenbock ('39) from sulfur balance studies with rats.

The methionine retention was much better than the cystine retention. Part of the poor cystine retention may have been exaggerated because of shedding of feathers, some of which became mixed with the droppings. Most of these, however, were separated before weighing and grinding the droppings.

It was expected that there would be considerable oxidation of cystine and methionine to sulfate but this was not the case, since there was less sulfate sulfur in the droppings than in the feed, indicating a retention of some of the sulfate sulfur. However, it is possible that some sulfate sulfur was lost from the droppings through bacteriological action.

Methionine may act as a source of methyl groups to replace choline (du Vigneaud, Cohn, Chandler, Schenck, and Simmonds, '41), so that in a deficiency of choline, methionine may supply methyl groups. To the extent that choline replaces methionine in this capacity, it probably replaces methionine in the diet.

#### SUMMARY

The nutritive value of the proteins of raw soybean oil meal, as determined by total gain in weight and gain per gram of protein consumed by chicks, was increased by autoclaving the meal at 100°C., 110°C., or 120°C. for 30 minutes. The protein nutritive values were lower when soybean oil meal was

autoclaved at 130°C. for 30 or 60 minutes than when it was autoclaved at 100°–120°C.

The availability for growing chicks of the methionine and cystine or the organic sulfur in soybean oil meal was increased by autoclaving the raw meal. The availability of the methionine and cystine or the organic sulfur was not as great when the meals were autoclaved at temperatures above 120°C. as it was when the meals were autoclaved at 100°C., 110°C., or 120°C. Added methionine increased the growth and feed efficiency on all diets. The addition of 0.2% dl-methionine to the basal diet increased the retention by the growing chick of the methionine originally in the unsupplemented diet. The methionine of the soybean oil meal was retained to a much greater extent than the cystine.

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# THE CAROTENE CONTENT OF CUBAN FOODS

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The carotene content of tropical plant and animal materials is not yet well known. The variety and exuberance of the flora and fauna of the tropics suggest the likely possibility of finding excellent sources of various members of the vitamin group including carotene. This is supported by the recent report of Cravioto, Lockart et al. ('45) on analyses of Mexican foods. These authors have recorded the exceptional nutritive value of some less commonly used foods such as the malvas for example. This report has encouraged us in our work of analysing Cuban foods, work which was begun long before the above mentioned report was published. In this paper we present the results of carotene analyses of seventy-five samples of forty-three Cuban foods, several of which, to the best of our knowledge, have never been analysed before.

## METHODS AND MATERIALS

As chromatographic separation of the carotenes from pigmented impurities has been demonstrated to be more specific than the commonly used biphasic purification, we chose a technique of the first type, namely the method of Wall and Kelley ('43). Except in the case of cacao, their modification for fresh material was employed. The use of a regulator for controlling the speed of the Waring blender was found necessary in many instances to avoid splashing and subsequent loss of carotene. This happened particularly in samples of soursop, canistel and cashew nut.

All of the samples were secured from the Havana curb markets, and were of the same quality and degree of ripeness as the food commonly consumed. Whenever possible the variety was recorded and two or three determinations of the same food were made. In the case of mango eight varieties were assayed. Only the edible part of the fruits was considered.

When extraction in the blender resulted in white extracts the result was reported as carotene-free. It should be noted that a yellow extract sometimes contained non-measurable carotene, as coloring impurities had been retained by the chromatographic column; this was true for samples of peanut oil and grapefruit. When a final extract of a 100-gm sample was concentrated to 10 ml, and gave a photometric reading of more than a 91% transmission, the sample was also reported as carotene-free.

The malanga, mamoncillo and pitahaya samples were hard to filter through fritted glass while that of mango was the easiest. Filtration was accelerated when a funnel with a capacity that was several times the volume to be filtered was employed.

We used Kimble amber and Pyrex low actinic glasses, although these are not specified for carotene determinations. We found that both colored glasses, especially the Pyrex red, made it difficult to observe the emulsions that were frequently encountered.

#### RESULTS AND DISCUSSION

The results of our analyses are presented in table 1. Examination of the table reveals that the mango, mamey de Santo Domingo, canistel and red cashew nut are the Cuban grown fruits richest in carotene. Next to these come the muskmelon, tangerine, Johnson banana, guava, red mamey and Jamaica cherry. Fair values were found for red pepper, yellow malanga and ripe plantain. Except for the Placero tomato, the rest of the foods analysed were found to be unimportant with respect to content of carotene. Soursop and coconut

were completely devoid of the pro-vitamin, while peanut oil, prú and grapefruit contained only non-measurable amounts.

The most interesting finding was the remarkably high carotene content of certain varieties of mango, values which prove to be the highest ever recorded among fruits. Of the eight varieties assayed, the Bizcochuelo and the Mulgoba showed very high values, while the others had about the same content as has been found in other countries. In one sample of Moro mango 77% of its crude carotene was found to be beta-carotene. A single Moro, Bizcochuelo or Mulgoba mango contains several times the daily allowance for adults of vitamin A recommended by the Food and Nutrition Board of the U. S. National Research Council ('43). In this connection it is of some interest to mention that Valledor and Fernández Flores ('40) and Rosenkranz and Vieta ('45) have found the mango to be a good source of vitamin C as well.

A comparison of our figures with those of Booher, Hartzler and Hewston ('42) and of Cravioto et al. ('45) shows that Cuban fruits are, in general, richer in carotene than the corresponding American and Mexican fruits. The values for tomato, red pepper, lemon, grapefruit, honey, potato, cucumber, watermelon and banana agree with those found for the same foods grown and analysed in the United States, and similarly, in the case of red mamey, sugar cane, guava, fig, lime, lemon, and some mango varieties with those for these foods grown and assayed in Mexico. The only difference between the American assays and ours pertain to papaya, and between the Mexican data and ours with respect to the banana. Climate, botanical variety, variation among samples, and no doubt other factors may be responsible for this. Appreciable variation in carotene content among different samples was experienced in many cases, but this is of frequent occurrence in food analyses, especially when common market samples are used as was the case in our study. It should be pointed out that the particular method of analysis employed by us is highly specific.



TABLE 1  
*Carotene content of Cuban foods.*

COMMON NAME	BOTANICAL NAME	VARIETY	CAROTENE <i>mg/gm of material</i>
Banana	Musa sapientum, Lin.	Johnson	2.4
Banana	Musa sapientum, Lin.	Manzano	0.3
Bee's honey			0.02
Cacao, raw	Theobroma cacao, Lin.	Baracoa	1.7
Cacao, toasted	Theobroma cacao, Lin.		2.1
Canistel	Lucuma nervosa, A.D.C.		9.5
Canistel	Lucuma nervosa, A.D.C.		74.0
Cashew nut	Anacardium occidentale, Lin.	Red	4.2
Cashew nut	Anacardium occidentale, Lin.	Red	9.5
Cashew nut	Anacardium occidentale, Lin.	Red	15.5
Coconut, flesh <sup>1</sup>	Cocos nucifera, Lin.		0.0
Coconut, flesh <sup>1</sup>	Cocos nucifera, Lin.		0.0
Coconut, milk <sup>1</sup>	Cocos nucifera, Lin.		0.0
Coconut, milk <sup>1</sup>	Cocos nucifera, Lin.		0.0
Cucumber	Cucumis sativus, Lin.		0.2
Chayote	Sechium edulis (Jacq.) S.W.	Spiny	0.03
Cherry, Jamaica	Malpighia glabra, Lin.		1.3
Fig	Ficus carica, Lin.		0.4
Fig	Ficus carica, Lin.		0.6
Grapefruit, juice <sup>2</sup>	Citrus decumana, Lin.		0.0
Grapefruit, juice <sup>2</sup>	Citrus decumana, Lin.		0.0
Guagüi	Colocasia antiquorum, Schott		0.2
Guava	Psidium guajaba, Lin.		1.6
Guava	Psidium guajaba, Lin.		2.8
Lemon, common	Citrus limonum, Risso		0.04
Lemon, sour (rind)	Citrus medica, Lin.		0.02
Lime, juice <sup>2</sup>	Citrus limetta, Risso		0.0
Lime, juice	Citrus limetta		0.02
Malanga <sup>2</sup>	Xanthosoma sagittifolium, Schott	White	0.0
Malanga	Xanthosoma sagittifolium, Schott	Yellow	10.8
Mamey, red	Achras zapota, Lin.		1.4
Mamey, red	Achras zapota, Lin.		2.4
Mamey de Sto. Domingo	Mammea americana, Lin.		11.7
Mamey de Sto. Domingo	Mammea americana, Lin.		14.0
Mamey de Sto. Domingo	Mammea americana, Lin.		52.0
Mamoncillo	Melicoea bijuga, Lin.		0.03
Mango	Mangifera, indica, Lin.	Bizeochuelo	41.5
Mango	Mangifera, indica, Lin.	Bizeochuelo	72.5
Mango	Mangifera, indica, Lin.	Filipino	9.5

TABLE 1 (Continued)

COMMON NAME	BOTANICAL NAME	VARIETY	CAROTENE <i>mg/gm of material</i>
Mango	Mangifera, indica, Lin.	Julián	19.0
Mango	Mangifera, indica, Lin.	Manga amarilla	20.8
Mango	Mangifera, indica, Lin.	Mango de puerco	16.4
Mango	Mangifera, indica, Lin.	Moro	115.0
Mango	Mangifera, indica, Lin.	Moro	139.5
Mango	Mangifera, indica, Lin.	Moro	164.0
Mango	Mangifera, indica, Lin.	Mulgoba	51.6
Mango	Mangifera, indica, Lin.	Toledo	16.4
Muskmelon	Cucumis melo, Lin.		7.6
Okra	Hibiscus esculentus, Lin.		2.4
Orange, sour (juice)	Citrus aurantium, Lin.		0.3
Orange, sour (juice)	Citrus aurantium, Lin.		1.3
Papaya	Carica papaya, Lin.	Yellow	0.6
Peanut oil <sup>1</sup>			0.0
Pepper	Capsicum annum, Lin.	Common red	27.6
Pitahaya	Selenicereus grandiflorus (L.) B and R		0.05
Plantain, ripe	Musa paradisiaca, Lin.	Burro	9.2
Potato	Solanum tuberosum, Lin.		0.02
Prá <sup>2</sup>			0.0
Sapodilla	Sapota achras, Mill.		0.2
Sapodilla	Sapota achras, Mill.		1.2
Sirup, sugar cane			0.03
Sirup, sugar cane			0.04
Soursop <sup>2</sup>	Annona muricata, Lin.		0.0
Soursop <sup>2</sup>	Annona muricata, Lin.		0.0
Sugar cane	Saccharum officinarum, Lin.		0.2
Sweetpotato	Ipomoea batatas, Lin.	White	2.1
Tamarind	Tamarindus indicus, Lin.		0.3
Tamarind	Tamarindus indicus, Lin.		0.8
Tangerine, juice	Citrus nobilis, Lour.		7.5
Tomato	Lycopersicum esculentum, Will.	Placero	5.8
Watermelon	Citrullus citrullus (L.) Karst		0.5
Watermelon	Citrullus citrullus (L.) Karst		1.3
Watermelon	Citrullus citrullus (L.) Karst		1.3
Yam, white	Dioscorea alata, Lin.		0.03
Yam, yellow	Dioscorea cayenensis, Lin.		0.1

<sup>1</sup> A 100-gm sample rendered a white extract in the blender.

<sup>2</sup> Final extract of a 100-gm sample concentrated to 10 ml gave a valueless photometer reading.

<sup>3</sup> A 10-ml sample gave the same results as in <sup>2</sup>.

## SUMMARY

The carotene content of seventy-five samples of forty-three Cuban plant foods was determined by the chromatographic technique of Wall and Kelley. Of the eight mango varieties assayed, three gave the highest values ever recorded among fruits. These values were higher than those for carrots and similar to the highest reported for spinach, both vegetables being analysed in the United States. Of the remaining mango varieties, several gave values similar to those reported from other countries. In one sample of the Moro variety 77% of the carotene proved to be beta-carotene. This variety had 115, 139.5 and 164  $\mu\text{g}$  of carotene per gram of pulp, respectively, in three samples. In general Cuban fruits are richer in carotene than the American and Mexican grown varieties.

The mango, canistel, mamey de Santo Domingo and red cashew nut had the highest values among Cuban fruits. A listing of the remaining "good" foods in order of decreasing carotene content gives the following: red pepper, yellow malanga, ripe plantain, muskmelon, tangerine, Placero tomato, guava, red mamey, okra and Johnson banana. Other foods would be rated appreciably below these; and the soursop, coconut, prú, grapefruit and peanut oil either lack carotene or have it in non-measurable amounts.

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# THE EFFECTS OF ADDED VITAMIN A ON THE CONJUNCTIVA AND THE LEVEL OF VITAMIN A IN THE BLOOD

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## ONE FIGURE

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Kruse ('41) reported that 99% of 166 adults in a low income group showed diminished transparency of the bulbar conjunctiva which he claimed was due to avitaminosis A. In 39% of his subjects elevations or spots which could be seen with the naked eye were present. Following the administration of 100,000 I.U. of vitamin A daily for 8 months to 70 adults, Kruse reported the following results:

"One person with spots has been completely restored and discharged. In all others with spots, the conjunctiva has become less vascular, thinner, clearer and more lustrous. The spots are much diminished in size; in many no longer grossly elevated; in some detectible only by microscope. Of the persons with 'nonspot' lesions, eight have been fully restored and discharged. Naturally, since the 'nonspot' lesions are usually less severe, more in this group were among the first to show complete recovery. Nevertheless, they have required not less than 6 months' intensive therapy.

"In both groups those who have not received therapy have shown no improvement."

## EXPERIMENTAL

In the following test, forty pupil nurses, who needed 1 to 2 years more to complete their training, agreed to act as sub-

<sup>1</sup> Under the direction of Alan Brown, M.D., F.R.C.P. (Lond.).

jects. Thirty-four of the forty were 19 to 21 years of age. Five were 22 to 23 years old; one was 26 years of age. Their general health was good and their physical examinations at the beginning of their training showed no defects. As far as was known they came from families that were comfortably off financially.

### *Initial examination of conjunctiva*

The bulbar conjunctiva, which is relatively transparent, contains numerous small blood vessels. It is attached by means of lax connective tissue to the opaque, firm sclera (Fuchs, '24). That it is freely movable on the sclera can be demonstrated by having the patient blink gently while observing the conjunctiva through a slit lamp. Another method of showing the mobility of the conjunctiva is to pull down the lower lid. During either of these procedures the blood vessels in the conjunctiva can be seen to move. The deep conjunctival blood vessels which are presumably attached to the outer surface of the sclera do not move (Wolff, '40). Using the slit lamp, Kruse ('41) described changes in both the superficial and deep parts of the conjunctiva, which he described as transparent, translucent or opaque according to their ability to transmit light. The present authors were unable to see any difference in transparency between the deep and the superficial parts of the conjunctiva. Consequently it was not possible to follow Kruse's method exactly. Instead, the transparency of the conjunctiva was estimated by the clearness with which the deeper, non-movable blood vessels could be seen through a slit lamp. The method used in this study was planned to demonstrate the conjunctival changes described by Kruse.

When examined with the slit lamp, all of the subjects showed some thickening of their bulbar conjunctivae. If one accepts Kruse's ('41) hypothesis, this thickening has occurred because these young women have suffered from a deficiency of vitamin A sometime prior to the examination. In other words,

100% of these subjects showed signs of avitaminosis A, according to Kruse.

As a result of this first examination with the slit lamp, the subjects were divided into three groups depending on the transparency of their bulbar conjunctivae. Group 1 had fairly clear conjunctivae. There were fifteen subjects in this group and five of them showed elevations or pingueculae in one or more of the limbic areas, at the equator (fig. 1). These elevations are raised areas roughly circular or triangular in outline. The elevations, according to Fuchs ('24) are due chiefly to an increase in the number and size of the elastic fibers. In some cases they are of a yellow color, due to the presence of a yellowish hyaline substance. These elevations are thought by ophthalmologists (Parsons, '38) to be more common in individuals exposed to much dust, wind, or other such conditions (see below).

Group 2 had somewhat more opaque conjunctivae and of the thirteen subjects in this group, eight showed elevations.

Group 3 had still more opaque conjunctivae and ten of the twelve subjects in this group showed elevations. Three of these individuals had three to four elevations each, which was more than any of the subjects in groups 1 or 2 showed.

None of the subjects showed markedly opaque conjunctivae or deeply pigmented elevations. Thirty-nine per cent of Kruse's patients showed one or more elevations that were visible to the naked eye, while in the present series, 57% showed such elevations. Kruse's subjects varied in age from 17 to 65 years, and were from low income groups, while in this series they were from 19 to 26 years old and were from higher income groups.

#### *The relation of outdoor sports to the presence of elevations*

To check roughly whether there was any relation between indulgence in outdoor sports and the presence of elevations, the subject was asked whether she had gone in for outdoor



sports freely or whether she had taken little of such exercise before coming into training. Then it was determined whether elevations were commoner in the girls who had taken much outdoor exercise and conversely whether the girls who did not indulge in much outdoor exercise rarely had conjunctival elevations. This seemed to be true in general — as 70% of the girls who took much outdoor exercise showed elevations, whereas of the girls who did not indulge in such exercise to any extent only 20% showed elevations. These figures suggest that exposure to wind and the other elements favors the development of these elevations.

#### *Amount of vitamin A in the meals*

The nurses received palatable meals which were planned by a dietitian, who held a university degree in Home Economics. As far as could be ascertained no reliable method for determining the total amount of both vitamin A and carotene in the meals was available, but the carotene alone could be fairly readily assayed by a method suggested by Jackson ('43). Carotene determinations were therefore made on 3 successive days each month for the last 9 months of the study. As was expected, the results of the carotene assays showed considerable variation. On 9 of the days the amount of carotene varied from 492 to 1000  $\mu\text{g}$ ; on 9 other days it ranged from 1000 to 5000  $\mu\text{g}$ ; on 10 days it was between 5000 and 25,000  $\mu\text{g}$ . When these figures are expressed in terms of vitamin A, using the conversion figure of 0.6  $\mu\text{g}$  carotene as equivalent to 1 I.U. of vitamin A, they are as follows: 820 I.U. to 1666 I.U.; 1666 I.U. to 8330 I.U.; 8330 I.U. to 41,650 I.U. The vitamin A Sub-Committee of the Accessory Food Factors Committee (Lister Institute and Medical Research Council) have recently (1945) reported tests on human beings in which only 25 to 40% of the carotene ingested in the form of green or yellow vegetables was retained in the body. It is probable therefore that about one-third of the carotene that is ingested is available to the body.

As we were not able to measure the vitamin A in the food by chemical methods, we estimated, using recent food tables, the amount of it present in the milk, cream, butter, cheese, eggs and liver eaten by a nurse on the same days. The amount of vitamin A in these foods varied from 2500 to 6000 I.U. per day, with an average of 4000 I.U., except on 3 days when it ranged from 12,000 to 14,000 I.U., due to the fact that liver had been served. These figures are approximations, at best, as even the figures given in the food tables show great variations, but they indicate that the meals contained good amounts of vitamin A. Therefore if the conjunctival changes are due to a deficiency of vitamin A, this must have occurred some time previously.

### *Dosage of vitamin A*

Half of each group was given 50,000 I.U. of vitamin A<sup>2</sup> daily in two capsules. Usually half of this was taken at breakfast and the remainder at bedtime. Occasionally the two capsules were taken together at bedtime. The other half of each group received identical looking capsules containing corn oil.<sup>2</sup> Care was taken to see that in each group, half, as nearly as possible, of the subjects with elevations received therapy. All of the nurses were under the impression that they were receiving vitamin A. The authors, who carried out all the examinations, did not know which subjects were receiving vitamin A and which were not. A responsible assistant distributed the capsules every 2 months. The nurses were reminded frequently — that is at least once every few weeks — of the importance of taking the capsules regularly. They also kept records of the number of capsules missed. In regard to the latter, during their annual 3 weeks' holiday and during the 12% of their time that they spent on night duty, the capsules were taken irregularly. During the rest of the year the nurses took them quite faithfully. About 40% reported that they took them very regularly; about 50% missed about

<sup>2</sup> We are indebted to Mead Johnson and Company, for supplies of this material in the form of gelatin capsules.

two per week; and about 10% forgot from three to six capsules per week. However even though some of the nurses forgot to take a fair number of the capsules, the total amount of vitamin A ingested during the 2 years of the test was very great.

### *Vitamin A blood levels*

Blood vitamin A values were determined in thirty of the subjects using the method of May, Blackfan, McCreary and Allen ('40). The first blood samples were taken after the test had been in progress for 9 to 10 months and the blood was drawn in the morning, 2 to 4 hours after the nurse had taken her capsule. The average level in the therapy group was about 9 blue units higher than in the control group (table 1, column 3) — no doubt due to the 25,000 I.U. of vitamin A which had been taken a few hours previously.

TABLE 1  
*Blood vitamin A values in blue units.*

SUBJECTS	NUMBER OF SUBJECTS	AFTER 9-10 MOS. THERAPY		AFTER 22-23 MOS THERAPY	
		Average	Usual range	Average	Usual range
Taking vitamin A	14	26.8 <sup>1</sup>	21 -33 <sup>1</sup>	22.3 <sup>2</sup>	14-30 <sup>2</sup>
Controls	16	17.9 <sup>1</sup>	12.4-21.5 <sup>1</sup>	21.4 <sup>2</sup>	14-25 <sup>2</sup>

<sup>1</sup> Blood was drawn 2 to 4 hours after 25,000 I.U. of vitamin A had been ingested.

<sup>2</sup> Blood was drawn 11 to 13 hours after 25,000 I.U. of vitamin A had been ingested.

In the second test, after 22 to 23 months on therapy, the nurses were asked to omit the capsule on the morning on which they were to be bled. Consequently the blood was drawn 11 to 13 hours after the last dose of vitamin A had been taken. As is shown in column 5 of table 1, the average blood vitamin A levels of the subjects on therapy and of the controls were very similar, as were also the ranges of the readings. It appears therefore that the addition of 50,000 I.U. of vita-

min A to the daily diet for a period of 22 to 23 months does not raise the blood vitamin A level except for the first few hours after a dose has been taken.

### *Technique of slit lamp examination*

Except during the months of July and August, each nurse was very carefully examined with the slit lamp approximately every 2 months, always by the same observer. Each examination required about 45 minutes' time for each nurse. The subject who was seated in front of the window, but not in direct sunlight, was first examined with the naked eye. The evenness of the surface of the conjunctiva, the number, position, shape and color of the elevations, any enlargement or engorgement of the plica and the extent of the vascular network were recorded. No regular changes in any of these tissues could be observed. This result is contrary to that of Kruse ('41) who described such changes following the ingestion of large amounts of vitamin A daily.

A large scale drawing (8" x 6") was made of all the deep bulbar conjunctival vessels that could be seen through the slit-lamp (fig. 1). The fact that the deep vessels do not move when the subject blinks or the observer pulls down the lower lid quickly, allows one to differentiate between the deep and the superficial vessels. The upper lid was held up and the lower lid held down by the subject so that all of the bulbar conjunctiva could be examined. The clarity with which the deep vessels could be seen and the number of them that were visible were used as a measure of the transparency of the conjunctiva. The clearness with which the various parts of the vessels could be seen was indicated by means of numbers written beside the vessels. The clearest parts were designated as 1; those parts just visible as 4, with gradations between designated 2 and 3. After each examination the drawing was traced on a thin sheet of paper — the vessels being shown in red (solid lines in fig. 1) and no figures being copied. At the next examination the vessels seen through the slit lamp were

compared with those shown in the drawing and the clearness with which they were now seen was marked in again by figures. Any new vessels seen at this examination were drawn in with a blue pencil (shown by dotted lines in fig. 1). Any vessels previously recorded and not seen on this examination were surrounded by blue circles and these were omitted in the next tracing. The aim of these drawings was to demonstrate whether the conjunctiva was becoming more transparent and by drawing in the vessels, one could be sure of their loca-

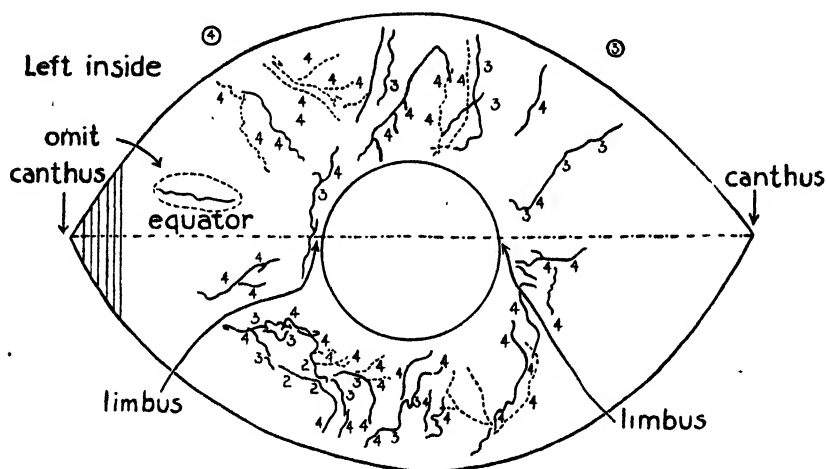


Fig. 1 Large scale drawing of deep conjunctival vessels; the examiner could not see the blood vessels enclosed by the dotted circle at this particular examination, although these vessels were seen in this area in the previous examination. Therefore in the tracing that was made from this drawing these blood vessels were omitted. This tracing served as a guide at the next examination.

tion in the conjunctiva. The subject whose deep conjunctival vessels are shown in figure 1, belonged to the group which had the most opaque conjunctiva (group 3), and therefore few vessels were seen and only parts of two of the vessels were relatively clear (marked 2). No vessels were very clear.

Kruse ('41) states that the thickness of the conjunctiva can often be estimated by counting the number of vessels seen crossing over each other in each zone (area between limbus and canthus). This was therefore done and the number of

vessels seen crossing over each other was recorded above each canthus. These numbers are enclosed in circles in figure 1. For example, 4 means that four strata of vessels crossing over each other could be seen on the average in that zone. This was recorded in the successive examinations but no regular change was noted.

*Results of slit lamp examinations  
after beginning of therapy*

At the end of each examination, the record and the drawings of the subject's eyes were compared with her previous drawings and records and the observer decided from this evidence whether more or fewer deep vessels were seen, whether some of these vessels were seen more or less clearly, or whether there was no change.

If the deep vessels were seen more clearly or if more of them were visible, either of which findings would indicate that the conjunctiva was more transparent, the subject was said to be receiving vitamin A. After the whole group of subjects had been examined each time, the assistant, who knew which nurses were receiving vitamin A, checked the results, removed the nurses' names from the sheet and reported in what percentage of the subjects the diagnosis of therapy was correct. From her report it was evident that it was quite impossible to determine by the use of this method who was receiving vitamin A and who was not.

The results of all the slit lamp examinations on each subject were summarized to decide whether any change had occurred from the beginning. Six of the subjects could be kept in the test for only 1 year. Five were on therapy and in three of these the conjunctivae seemed clearer after the year of therapy. The sixth, who was a control, showed no change in her conjunctivae.

Thirty-four of the patients took therapy for 2 years and a summary of the slit-lamp examinations on them is shown in table 2. In the subjects of group 1 (clearest conjunctivae)

none of those on therapy showed clearing, whereas 43% of the controls apparently had clearer conjunctivae at the end than at the beginning of the test. Of the subjects in group 2 (moderately opaque conjunctivae) 40% of those on therapy showed some clearing, but 33% of the controls showed similar clearing. Of the ten subjects with the most opaque conjunctivae (group 3), none showed any clearing, whether they had taken vitamin A or not.

TABLE 2

*Percentage of subjects showing more transparent conjunctiva at end of test—determined from 8-9 slit lamp examinations on each subject.*

INITIAL APPEARANCE OF CONJUNCTIVA	SUBJECTS GIVEN VITAMIN A (2 YRS.)		CONTROLS	
	Number of subjects	% clearer	Number of subjects	% clearer
Group 1 — Clearest	6	0	7	43
Group 2 — Moderately opaque	5	40	6	33
Group 3 — Most opaque	5	0	5	0

Two of the control subjects showed no change consistently throughout the 2 years of the study. One of the subjects taking vitamin A showed progressive and regular clearing of her conjunctivae but her elevations did not disappear. In all the other thirty-one subjects who were studied for the full 2 years (fifteen on therapy and sixteen controls) the conjunctiva on some examinations seemed to have become more transparent, but on subsequent examinations it seemed to show no change or to have become less transparent.

If the conjunctival thickening is caused by a lack of vitamin A, one would certainly expect that the administration for 2 years of enormous daily doses (50,000 I.U.) of vitamin A would cause the conjunctivae to become definitely thinner. The conjunctivae did not become clearer in these subjects and one would therefore conclude that the thickening was not due to a deficiency of vitamin A.

*Photographic studies of conjunctiva*

A kodachrome photograph, approximately life size, was taken of each subject's eyes every 6 months. All the conditions, i.e., camera, type of film, flash bulbs, etc., were identical as far as could be determined on each occasion. However the photographs taken at different times varied greatly in their color tones, due to uncontrollable variations in the films and their processing. It was therefore very difficult to compare the pictures taken at different times. Nevertheless at the end of the test after a study of the complete series of pictures of each of the subjects both in a viewing box and by projection in pairs on a smooth screen, a tentative conclusion was reached as to any change in the transparency of the conjunctiva. It

TABLE 3

*Percentage of subjects showing more transparent conjunctiva at end of test — as shown by photographs.*

INITIAL APPEARANCE OF CONJUNCTIVA	SUBJECTS GIVEN VITAMIN A	CONTROLS
	%	%
Group 1 — Clearest	33	43
Group 2 — Moderately opaque	60	66
Group 3 — Most opaque	40	60

is felt, however, that the photographic records are of doubtful value because of the technical difficulties involved. The results are shown in table 3. No difference could be detected between the nurses on therapy and the controls.

*Time lost due to infections*

While this study was in progress a record was kept of the number of days the nurses were off work due to illness during the 2 years (table 4). Seven of the sixteen subjects on vitamin A therapy, or 44%, lost time from work because of respiratory infections during the 2 years of the test. These nurses suffered from a total of fifteen infections, which resulted in a loss of 109 working days.



WITH VITAMIN A				WITHOUT VITAMIN A			
Total no. of nurses given vitamin A 16 No. of these nurses with respiratory infections 7 Per cent of these nurses with respiratory infections 44 Time lost because of respiratory infections — days 109 Number of infections 15				Total no. of nurses serving as controls 18 No. of these nurses with respiratory infections 11 Per cent of these nurses with respiratory infections 61 Time lost because of respiratory infections — days 198 Number of infections 24			
DETAILS OF RESPIRATORY INFECTIONS IN NURSES GIVEN VITAMIN A				DETAILS OF RESPIRATORY INFECTIONS IN CONTROL NURSES			
Name	No. infections	Time off <i>days</i>	Diagnosis	Name	No. infections	Time off <i>days</i>	Diagnosis
Au	1	5	"Flu"	Ea	2	30	"Flu" (fever 2 wks.)
Ec	2	12	Sore throat, cough			7	"Flu"
		1	Cold	Br	3	14	Bronchitis
Jk	3	2	Cold		4	4	Cold
		2	Cold		13	13	Sinusitis
		6	"Flu"	Ed	1	3	Tonsillitis
N	1	8	Cold	G	2	5	Cold
R	5	13	Strept. throat		2	2	"Flu"
		9	Cold	Ho	2	1	Cold
		11	Swollen cerv. glands		1	1	Cold
		10½	Tonsillitis	Pk	1	7	Cold
		13	Strept. throat	M	4	2	Cold
T	1	5	Cold			7	"Flu"
Ew	2	11	Bronchitis			14	"Flu"
		10	Cold (fever)		15	15	Cold
				Rc	1	14	"Flu"
7 pts.	15 infec.	109 days		Ry	1	6	Cold
				Sch	4	8	"Flu"
					9	9	Tonsillitis, sinusitis
					18	18	Cold
					4	4	Cold
				Tu	3	6	Cold
					3	3	Laryngitis
					5	5	Cold

The records of the control group (no vitamin A given) were less good. Eleven of these 18 nurses, or 61%, were off duty because of respiratory infections. They suffered from twenty-four respiratory infections, necessitating 198 days off duty.

No record was kept of the slight respiratory infections which did not put the nurses off work.

As for non-respiratory infections, the difference between the two groups was slight. Four nurses in the group receiving therapy lost 50 days' time; seven control nurses lost 59 days' time. These infections included appendicitis, conjunctivitis, food poisoning, "digestive upsets," mumps and infected fingers.

The group receiving therapy did show a lower incidence of incapacitating respiratory infections but the numbers are so small that the results are not conclusive.

#### CONCLUSIONS

1. The eyes of forty healthy young pupil nurses were examined with a slit-lamp; 100% of them showed thickening of the bulbar conjunctivae. In other words, if one accepts the hypothesis of Kruse ('41), all of these nurses had suffered from avitaminosis A at some time prior to this examination. The forty nurses were then divided into two groups as nearly alike as possible as far as the transparency of their conjunctiva was concerned. One group was given 50,000 I.U. of vitamin A per day in two divided doses. The other group received similar placebos containing corn oil. All of the nurses believed that they were receiving vitamin A and the observer did not know to which group any subject belonged. Thirty-four of the subjects took the capsules for a period of 2 years. Their conjunctivae were examined with a slit lamp at frequent intervals. With the use of this method it was not possible to determine which nurses were receiving vitamin A and which were not, except in the case of three individuals (two controls and one on therapy). In none of the subjects with conjunctival elevations at the beginning did therapy cause the elevations to

disappear. Therefore one would conclude that the conjunctival thickening is not caused by a deficiency of vitamin A.

2. Except for the first few hours after the 25,000 I.U. dose of vitamin A was taken, the blood vitamin A levels were similar to those in the control group.

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# THE NUTRITIONAL SIGNIFICANCE OF ANIMAL PROTEIN SUPPLEMENTS IN THE DIET OF THE CHICK

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It has long been believed that a good poultry diet could not be formulated without including a certain amount of animal protein feeds. That such animal products in the poultry diet are of distinct value is not doubted, but for some time, it has been a thought of ours that perhaps the contribution of these materials is not due so preponderantly to amino acids as has been supposed. More recently it has been established that, in addition to amino acids, minerals, and the better-known vitamins, certain of these animal protein supplements contribute nutritional elements to a practical chick diet, the nature of which is not understood. The indications are that these nutritional effects are due either to still unidentified substances or factors, vitamin-like in character, or to certain little-understood relationships or interactions involving known nutrient materials.

These findings have been an outgrowth of attempts to substitute the more readily available plant protein sources, such as soybeans, for the animal protein feeds which were scarce during the war years. Such attempts led first of all to a recognition of methionine deficiency in soybeans. It is now

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well known that methionine supplements a chick diet which contains soybean oil meal as the major source of protein (Hayward and Hafner, '41; Marvel, Carrick, Roberts and Hauge, '44). Almquist, Mecchi, Kratzer and Grau ('42) stated that heated soybean is slightly deficient in methionine for the chick at the 20% protein level, but is complete in respect to all other amino acids required by the chick.

The value of fish meal in supplementing such a diet is also well known. Christiansen, Deobald, Halpin and Hart ('40) reported that chick rations in which soybean oil meal furnished the majority of the protein produced exceptionally good growth when 3 or 4% fish meal was added. They reported that sardine meal appeared to be superior to menhaden or whitefish meal in this respect. Carver and Evans ('43) stated that a soybean chick starter diet was effectively supplemented by herring fish meal but not by meat scraps. Hammond and Titus ('44) reported that sardine fish meal is of outstanding value as a protein supplement to soybean meal, being superior to meat scrap or dried skim milk. They found that the protein of yeast failed to supplement the protein of soybean. Bird and Mattingly ('45) in tests designed to determine if methionine would supplement their diet as effectively as fish meal, found a significant improvement in growth of chicks when 0.2% dl-methionine was added to a starting and growing mash based largely on corn and soybean oil meal, and reported that the growth stimulus due to the methionine slightly exceeded that obtained by supplementation with 4% fish meal. They raised the question of the possible existence in fish meal of a substance or substances other than methionine, capable of performing, at least in part, the same biological functions as methionine. They did not, however, report feeding fish meal and methionine supplements together. Cravens, McGibbon, and Halpin ('45) found the addition of condensed fish press water or ground fish viscera to be highly effective in supplementing a diet composed of yellow corn, wheat by-products, meat scraps, soybean oil meal, minerals, fish oil and riboflavin.

During the course of research in this laboratory, we have had occasion to investigate certain phases of this problem. Our results are reported here.

#### EXPERIMENTAL

In an attempt to demonstrate the existence of an unidentified factor in fish meal, as indicated by work employing practical diets based largely on corn and soybeans, a purified diet was fed ad libitum to single-comb White Leghorn cockerels. These chicks were fed in groups of ten, in electrically-heated metal cages in an air-conditioned room. The percentage composition of the purified ration was as follows: casein, vitamin-free 18.0; gelatin 10.0; soybean oil 5.0; gelatinized starch 52.7; and gelatinized starch plus supplements q.s. to total 100. The supplements, in milligrams per 100 gm of ration, were as follows: NaCl, 836.8;  $K_2HPO_4$ , 1611.1;  $CaHPO_4 \cdot 2H_2O$ , 1374.7;  $MgSO_4$ , 249.0;  $CaCO_3$ , 1498.7;  $FeC_6H_5O_7 \cdot 6H_2O$ , 137.4;  $MnSO_4 \cdot 4H_2O$ , 40.0;  $ZnCl_2$ , 1.25;  $CuSO_4 \cdot 5H_2O$ , 1.5; KI, 4.0; l-cystine, 200.0; thiamine hydrochloride, 0.3; riboflavin, 0.6; pyridoxine hydrochloride, 0.4; dl-calcium pantothenate, 3.0; nicotinic acid, 10.0; 2-methyl-1,4-naphthoquinone, 0.5; i-inositol, 100.0; biotin, 0.015; choline chloride, 150.0. Halibut liver oil containing 60,000 U.S.P. units of vitamin A and 1,000 U.S.P. units of vitamin D per gram, fortified by the addition of 1,500 A.O.A.C. units of vitamin D<sub>3</sub> per gram and 10.5 mg alpha-tocopherol acetate per gram, was fed orally at the rate of 2 drops per week.

The results obtained on this purified ration are shown in table 1. The basal group failed to grow, as was expected, due to a lack of "folic acid." No improvement resulted from the addition of fish meal to the purified ration,<sup>3</sup> contrary to the growth benefits noted with a corn-soybean ration. As a positive control, yeast extract, which has been found to be of little benefit as a supplement to the corn-soybean ration, permitted good growth on the purified diet.

<sup>3</sup> Cravens, McGibbon and Halpin ('45) have since reported that condensed fish press water failed to improve growth on a purified diet.

In another part of our investigations, a semi-practical ration based largely on corn and soybean oil meal was employed, and various supplements were used in our attack upon the problem. The basal rations used are shown in table 2. All of these rations were calculated to contain 18% crude protein. Fat-soluble vitamins were fed orally at one-half the level used with the purified ration.

TABLE 1  
*Experiments with purified ration.*

SUPPLEMENTS	AVERAGE BODY WEIGHT			
	1 week	2 weeks	3 weeks	4 weeks
	gm	gm	gm	gm
<i>First experiment</i> <sup>1</sup>				
None	57.1	72.7	83.6	80.0 (8 dead)
Menhaden fish meal, 3%	54.2	69.5	83.2	84.5 (6 dead)
<i>Second experiment</i>				
None	66.0	87.8	80.0	(10 dead)
Sardine fish meal, 2%	65.8	93.8	110.5	(10 dead)
Sardine fish meal, 4%	62.4	85.6	104.3	(10 dead)
Yeast extract, 1%	73.6	121.8	185.2	268.8

<sup>1</sup> Experiments were carried out with groups of ten chicks for each ration.

TABLE 2  
*Basal rations used.*

RATION NUMBER	1	2	3	4	5
<i>Ingredients in per cent:</i>					
Ground whole yellow corn	69.5	71.1	72.5	71.1	72.5
Heated extracted soybean oil meal, 44% protein	26.0	22.4	19.0	22.4	19.0
Pacific sardine fish meal, 70% protein		2.0	4.0		
Casein-gelatin-cystine mixture				2.0	4.0
Corn starch plus supplements	2.0	2.0	2.0	2.0	2.0
Tricalcium phosphate	2.0	2.0	2.0	2.0	2.0
Iodized salt	0.5	0.5	0.5	0.5	0.5
<i>Supplements in mg per 100 gm ration:</i>					
Manganese sulphate	20.0	20.0	20.0	20.0	20.0
Riboflavin	0.3	0.3	0.3	0.3	0.3
Pyridoxine hydrochloride	0.3	0.3	0.3	0.3	0.3
2-Methyl-1,4-Naphthoquinone	0.04	0.04	0.04	0.04	0.04

## RESULTS

The first step in our experimentation was to determine the extent to which methionine was able to supplement the basal ration (Ration no. 1). In repeated trials with this diet, we have found that the maximum effect of methionine supplementation can be obtained with 0.15%, or at the most 0.30%,

TABLE 3  
*Effect of methionine on basal ration.*

RATION	ADDED METHIONINE	AVERAGE BODY WEIGHT			
		1 week	2 weeks	3 weeks	4 weeks
no.	%	gm	gm	gm	gm
1	0.0	64.4	113.2	178.7	247.1
1	0.15	68.4	121.2	198.8	291.8
1	0.30	68.4	122.6	202.2	301.8
1	0.60	70.2	124.2	200.8	301.4
1	1.20	67.4	111.8	170.8	241.8

TABLE 4  
*Results with methionine and fish meal.*

RATION	ADDED METHIONINE	AVERAGE BODY WEIGHT			
		1 week	2 weeks	3 weeks	4 weeks
no.	%	gm	gm	gm	gm
1		65.2	103.2	154.2	202.0
1	0.15	57.8	116.0	182.0	252.0
1	0.30	71.8	120.7	183.6	252.2
2		71.6	127.0	203.6	304.2
2	0.15	74.2	128.2	207.1	305.1
2	0.30	69.5	125.1	206.4	299.7
3		69.4	127.6	203.2	306.8
3	0.15	75.4	130.2	209.8	305.4
3	0.30	72.4	120.6	191.4	282.8

of dl-methionine; 0.60% was no improvement over 0.30%, and 1.20% methionine caused growth retardation. One such trial is presented in table 3. Consequently, in the subsequent trials with methionine and other supplements methionine has been fed at the 0.15 and 0.30% levels only.

The results of the next experiment, involving fish meal as well as methionine, are shown in table 4. In order to vis-



ualize more clearly the differences between total weight gains at 4 weeks due to methionine and fish meal as independent variables, the growth increment differences were obtained, by subtraction, from the 4 weeks' column of table 4, and are presented in table 5, column 1.

Since these increments show a beneficial effect of fish meal, even in the presence of an optimal level of methionine, the thought occurred to us that this beneficial effect might be due to the presence of known vitamins in the fish meal. In an attempt to rule out this possibility, the ration was supplemented with the following ingredients (milligram per 100

TABLE 5  
*Growth increments at 4 weeks.*

WEIGHT GAIN DUE TO 0.3% METHIONINE IN THE PRESENCE OF VARYING AMOUNTS OF FISH MEAL		
	Column 1 <i>gm</i>	Column 2 <i>gm</i>
Ration no. 1 (no fish meal)	+ 50.2	+ 42.6
Ration no. 2 (2.0% fish meal)	— 4.5	— 13.2
Ration no. 3 (4.0% fish meal)	— 24.0	— 1.4
WEIGHT GAIN DUE TO 2.0% FISH MEAL IN THE PRESENCE OF VARYING AMOUNTS OF METHIONINE		
	Column 1 <i>gm</i>	Column 2 <i>gm</i>
No methionine added	+ 102.2	+ 87.8
0.15% methionine	+ 53.1	+ 38.0
0.30% methionine	+ 47.5	+ 32.0

gm of ration): thiamine hydrochloride, 0.3; riboflavin, 0.6; pyridoxine hydrochloride, 0.4; dl-calcium pantothenate, 3.0; nicotinic acid, 10.0; 2-methyl-1,4-naphthoquinone, 0.5; i-inositol, 100.0; p-aminobenzoic acid, 3.0; biotin, 0.015; and choline chloride, 150.0. When this supplement was used, the riboflavin, pyridoxine and 2-methyl-1,4-naphthoquinone levels listed in the basal ration were omitted. Vitamins A, D<sub>3</sub>, and alpha-tocopherol were supplied orally as usual.

The results in the presence of this supplement were consistent with the previous results, as shown in table 5, column 2. This indicated that the effect of fish meal in all probability

was not due to any of the constituents included in the supplement, or methionine.

Our next step was to add 0.5% yeast extract<sup>4</sup> to the diet in addition to the supplement listed above. This was done in order to provide a source of folic acid activity, and also because Hill, Scott, Norris and Heuser ('44) had reported the possibility of a plant protein diet being deficient in unidentified factors supporting chick growth which could be supplied by feeding dried brewers' yeast. Our results with yeast extract are shown in table 6.

TABLE 6  
*Effect of yeast extract.*

RATION	SUPPLEMENTS	AVERAGE BODY WEIGHT			
		1 week	2 weeks	3 weeks	4 weeks
no.		gm	gm	gm	gm
1	Vitamins, choline	71.4	120.4	178.8	250.8
1	Vitamins, choline, 0.30% methionine	74.4	128.4	201.8	293.4
1	Vitamins, choline, 0.5% yeast extract	76.4	127.8	204.0	286.0
1	Vitamins, choline, 0.30% methionine, 0.5% yeast extract	72.4	125.0	198.8	289.2

We next attempted to determine the extent to which the amino acids present in the fish meal protein were responsible for its growth stimulation. Using the amino acid analysis from Block and Bolling ('45), by calculation it was determined that a mixture containing 1296 gm of vitamin-free casein, 144 gm of gelatin and 5 gm of cystine should provide the amino acids known to be required by chicks at levels approximately equal to those contained in fish meal. Such a mixture was prepared, and substituted for fish meal at equivalent levels in rations 4 and 5 (table 7).

<sup>4</sup>Standard Brands Type III.

TABLE 7  
*Effect of casein mixture.*

RATION	AVERAGE BODY WEIGHT			
	1 week	2 weeks	3 weeks	4 weeks
no.	gm	gm	gm	gm
1 (basal)	63.4	106.4	153.0	197.8
2 (2.0% fish meal)	66.6	118.8	202.8	294.6
3 (4.0% fish meal)	68.6	124.8	202.8	306.9
4 (2.0% casein mixture)	60.6	98.2	147.4	211.2
5 (4.0% casein mixture)	63.6	103.6	159.4	212.6

### DISCUSSION

From the data in tables 4 and 5 it appears evident that sardine fish meal supplements the basal diet more completely than methionine. In the presence of fish meal, added methionine was of little value, although methionine did supplement the diet, to a lesser extent, when fish meal was absent. On the other hand, a diet containing optimal levels of methionine, according to our findings, was still incomplete and benefitted materially from further supplementation with fish meal. This beneficial effect of fish meal occurred over and above any supplementary effect of methionine, choline, or any of the identified vitamins known to be required for chick growth.

From table 6 it appears that the addition of 0.5% yeast extract to the basal diet containing the vitamin mixture plus choline did exert some positive effect upon growth. However, a 0.30% methionine supplement likewise increased growth without yeast extract, and the presence of yeast extract in addition to methionine did not induce greater growth than that obtained with methionine alone. Hence we have concluded that the unknown factor present in sardine fish meal which supplemented the rations containing 0.30% methionine is probably not directly related to the unknown factor in brewers' yeast.

The casein-gelatin-cystine mixture (table 7) failed to increase gain in weight significantly above the basal level, whereas the fish meal levels fed as positive control groups repeated past performance. From this evidence it appears

doubtful if a deficiency of amino acids other than methionine is likely to be the first limiting factor in our basal ration. As further evidence, we have obtained, in preliminary work, a water-soluble fraction from sardine fish meal which effectively supplements the basal ration when added at low levels, as shown in table 8.<sup>5</sup> We have attempted to eliminate the probability that choline, methionine, known vitamins, or unknown factors present in yeast extract, are responsible to any major extent for the effect produced with sardine fish meal. Our trials with the casein mixture, and with the fish meal fraction, tend to indicate that we are dealing with an unknown factor,

TABLE 8  
*Effect of fish meal fraction.*

RATION	FISH MEAL FRACTION	AVERAGE BODY WEIGHT			
		1 week	2 weeks	3 weeks	4 weeks
<i>no.</i>	<i>%</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>
1		68.2	105.6	166.8	220.2
1	0.06	75.8	122.0	211.6	298.8
1	0.12	72.2	115.6	201.6	300.8
2		73.2	124.6	212.0	332.2
3		74.4	128.6	222.0	324.6

present in sardine fish meal, and required at the low levels commonly associated with vitamins rather than amino acids.

The results with the purified ration, however, are difficult to reconcile since it appears that the postulated unknown factor in fish meal is needed for growth on the corn-soybean basal diet but is of no value when added to a purified diet. It might be presumed that this failure was due to a primary lack of "folic acid" in the purified diet, which was not supplied by the fish meal, and because of this the secondary deficiency of another unknown factor did not manifest itself. This is hardly likely in view of the results with yeast extract, since it would be necessary to assume then that yeast extract

<sup>5</sup> Berry, Carrick, Roberts and Hauge ('45) have since reported that a water extract of fish press water containing only a small percentage of protein had most of the supplementary value of whole fish press water.

contained the same unknown factor (in addition to "folie acid") as fish meal and this did not appear to be the case from the feeding trials in which yeast extract was added to the corn-soybean ration. Another alternative is that the casein or gelatin could be a source of the unknown factor in the purified diet, but this hypothesis fails in view of the negative results obtained when casein and gelatin were used to replace fish meal in the corn-soybean diet.

A more likely explanation is that fish meal increases chick growth on a corn-soybean diet by supplying some factor, known or unknown, whose requirement has been augmented, or created, by the presence of corn and/or soybean oil meal in the diet. This would explain the failure to discover any such factor using the purified diet. If this should prove to be the case, the factor present in sardine fish meal would be of great practical importance nevertheless, since corn and soybeans are likely to continue to enjoy widespread use in poultry diets.

#### SUMMARY

A study was made of the extent to which dl-methionine and sardine fish meal are able to supplement a chick ration containing proteins from corn and soybeans only. Such a diet appears to be lacking in an unknown growth factor which is present in sardine fish meal. There is some evidence that the need for this factor is a peculiarity of the corn-soybean diet.

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# PANTOTHENIC ACID DEFICIENCY AND REPRODUCTION IN THE RAT<sup>1</sup>

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The importance of pantothenic acid for normal reproduction and embryonic development in the chick has been stressed by several investigators, e.g., Bauernfeind and Norris ('39), Gillis, Heuser, and Norris ('42), and Pearson et al. ('45). However, little is known concerning the effects of pantothenic acid deficiency on reproduction in rats. In general, it may be said that in chronic nutritional deficiencies with prolonged survival, the oestrous cycle is irregular or absent (Evans and Bishop, '22 a). In severe, acute deficiency in pantothenic acid it is known that the majority of animals do not mature before they die (Figge and Allen, '42). The study of reproductive phenomena in partial prolonged pantothenic acid deficiency has not, as far as we are aware, been reported by any investigator.<sup>2</sup>

If a pantothenic acid deficient diet is given to rats on the day of parturition, little interference with lactation is shown since 83% of the young are weaned.<sup>3</sup> Therefore, it seemed

<sup>1</sup> Aided by grants from the Board of Research and from the Department of Agriculture of the University of California, and the Rockefeller Foundation, New York City. The following materials were generously contributed: crystalline B vitamins from Merck and Company, Inc., Rahway, New Jersey; synthetic alpha-tocopherol from Hoffmann-LaRoche Company, Nutley, New Jersey.

<sup>2</sup> In this laboratory we have occasionally succeeded in breeding female rats deficient in the filtrate fraction of the B complex but failure of implantation or resorptions has always resulted (Evans and Emerson, unpublished).

<sup>3</sup> Nelson, M. M., and H. M. Evans — In preparation.



of interest to place normal female rats on pantothenic acid deficient diets at intervals during gestation or even before mating to see whether any upsets in the reproductive function would occur. Jukes ('40) mentioned the occurrence of resorptions in part of a group of stock rats placed on pantothenic acid deficient diets at mating. Sure ('41) noted that reproduction on purified diets without the addition of calcium pantothenate was very abnormal since either "sterility" of the mother or a high incidence of still births in the young resulted.

#### EXPERIMENTAL

Normal female rats, 2 to 5 months of age, were bred with normal males and placed on the experimental diets the first or the thirteenth day of gestation. Other groups of rats were maintained on pantothenic acid deficient diets for 15 days and then bred as rapidly as possible. Paired-feeding studies were carried out with some groups. Vaginal smears were examined during gestation for the presence of erythrocytes, the sign that implantation has occurred; all rats were weighed at regular intervals. The deficient and control groups were carefully balanced according to body weight and age at the time of breeding. All rats were kept in cages with screens until the day before littering was expected to occur.

Two basal deficient diets, differing only with respect to the levels of the B vitamins and to the composition of the salt mixture, were used. Both diets were composed of 24% alcohol-extracted casein, 64% sucrose, 8% hydrogenated cottonseed oil,<sup>4</sup> and 4% salts. The deficient diet 841 contained McCollum salts No. 185<sup>5</sup> and the following B vitamins per kilogram diet: thiamine HCl 2 mg, pyridoxine HCl 2 mg, riboflavin 5 mg, p-aminobenzoic acid 5 mg, nicotinic acid 10 mg, inositol 200 mg, and choline HCl 0.5 gm. The corresponding control diet 831 contained, in addition, 28 mg calcium pantothenate per kilogram diet. These vitamins were added to the diet in the form of a 20% alcoholic

<sup>4</sup> Crisco.

<sup>5</sup> McCollum and Simmonds ('18).

solution. The deficient diet 846 contained an improved salt mixture, salts No. 4,<sup>6</sup> and higher levels of the same B vitamins; thiamine HCl 5 mg, pyridoxine HCl 5 mg, riboflavin 10 mg, p-aminobenzoic acid 10 mg, nicotinic acid 20 mg, inositol 400 mg, and choline HCl 1.0 gm. The corresponding control diet 836 contained, in addition, 50 mg calcium pantothenate per kilogram diet. These vitamins were added to the diet as a dry mixture with sucrose. Fresh batches of all diets used were made up weekly, placed in dark bottles, and kept in the refrigerator except when the diet was being fed. Every deficient and control rat received weekly a fat-soluble vitamin mixture by stomach tube or in supplement cups that furnished a minimum of 400 U.S.P. units vitamin A, 58 A.O.A.C. Chick Units vitamin D, 3 mg synthetic alphatocopherol,<sup>7</sup> and 325 mg corn oil.<sup>8</sup>

*Experimental diets started the thirteenth day of gestation*

There was no significant difference in reproductive performance between the deficient and control groups as judged by the average number of young born and their average weight at birth (table 1).

*Experimental diets started the first day of gestation*

Marked upsets in the reproductive function were observed with this procedure. Approximately one-third of the rats in both deficient groups underwent resorptions instead of littering (table 1). This is in agreement with the findings of Jukes ('40). These resorptions were confirmed in each case at autopsy by finding several sites of resorption in the uterus. The remainder of the deficient rats littered but the average weight of the young at birth was significantly decreased. In the case of the rats maintained on the deficient diet 841, the average number of young per litter was also decreased. In

<sup>6</sup> Hegsted et al ('41).

<sup>7</sup> Hoffmann-LaRoche.

<sup>8</sup> Mazola.

TABLE 1  
*Effect of pantothenic acid deficiency on reproduction.*

EXPERIMENTAL GROUP	DIET NO.	NO. OF RATS BREED	AGE WHEN BREED	FAILED IMPLAN-TATION	RESORP-TIONS OF IMPLAN-TATIONS		LITTERS OF IMPLAN-TATION	TOTAL NO. YOUNG	YOUNG BORN DEAD	AV. SIZE OF LITTER	AV. WT. YOUNG
					%	%					
PANTOTHENIC ACID DEFICIENT	13th day gestation	9	92 (89-123)	0	0	0	100	83	0	9.2(5-12)	5.4
		10	99 (70-126)	0	0	0	100	87	0	8.7(4-11)	5.9
	1st day gestation	10	112 (85-159)	0	0	30	70	48	6	6.9(6-8)	4.8
		11	128 (103-155)	0	0	36	64 <sup>2</sup>	61	10	8.7(4-13)	4.6
PANTOTHENIC ACID CONTROLS	16-23 days before gestation	11	129(117-142)	27	50	50 <sup>3</sup>	0	27	4	6.8(5-9)	4.3
		9	137(123-131)	33	100	0			..		
	13th day gestation	10	88 (69-123)	0	0	0	100	89	0	8.9(7-11)	5.6
		11	98 (70-126)	0	0	0	100	104	0	9.5(8-12)	6.3
	1st day gestation	11	116 (86-159)	0	0	0	100	102	1	9.3(4-15)	5.5
		10	121 (96-134)	0	0	0	100	91	11 <sup>4</sup>	9.1(3-15)	5.5
	16-23 <sup>1</sup> days before gestation	10	132(120-143)	10	0	0	100	72	6	8.0(3-10)	5.9
		10	130(118-143)	0	0	0	100	83	0	8.3(4-13)	5.7

<sup>1</sup> These rats were maintained on the deficient diet for 16-23 days before breeding; control diets were started the day of breeding.

<sup>2</sup> Two of these seven litters were found at autopsy; they consisted of some living fetuses, some dead and others in the process of resorption.

<sup>3</sup> One of these four litters was found at autopsy; it consisted of some living fetuses, some dead and others in the process of resorption.

<sup>4</sup> These dead young were in one litter only.

the group on the deficient diet 846 two of the seven litters were found in the uterus at autopsy and consisted of some living fetuses, some dead, and others in the process of resorption.

*Experimental diets started 16-23 days  
before breeding*

In this experiment all rats were placed on the deficient diets for 15 days. Then they were bred as fast as possible with normal males. Half of them were continued on the deficient diets and the other half placed on the control diets the day of breeding. The average length of time for all rats on the deficient diet before breeding was 18 days for diet 841 and 19 days for diet 846.

Approximately one-third of the deficient rats failed to implant (table 1), thus indicating a marked interference with the reproductive mechanism. Of the rats maintained on diet 841, 50% of the implantations underwent resorption, while 100% of the implantations on diet 846 resorbed. This was confirmed in each case by examination of the uterus at autopsy. Of the four litters that were obtained from rats maintained on diet 841, one of them was found in the uterus at autopsy and consisted of some living fetuses, some dead, and others in the process of resorption. Furthermore, the average number of young per litter and their average weight at birth were significantly decreased in comparison with control values.

In the "control" rats, which received pantothenic acid on the day of breeding, the only indications of damage to the reproductive mechanism from maintenance on the deficient diets for 2 to 3 weeks before breeding were of doubtful significance, i.e., the slightly decreased number of young per litter and the failure of implantation for one rat maintained previous to breeding on diet 841.

*Effect of limited dietary intake on gestation*

To eliminate the factor of undernutrition, pair-fed controls were added to the previous experimental procedure. Of the

rats maintained on the deficient diet 841 for 15 days before breeding, one-third of them were continued on the deficient diet and their dietary intake measured daily; one-third of them received 1 mg calcium pantothenate daily plus the same quantity of diet 841 consumed by the corresponding deficient rats on the same day of gestation; and the remaining one-third received 1 mg calcium pantothenate daily plus diet 841 ad libitum. The rats were carefully paired in respect to age and body weight on the day of mating and with regard to the weight change during the first 15 days on the deficient diet. To guard against the possibility of a vitamin E deficiency, the quantity of fat-soluble vitamins given weekly was doubled.

The experimental data are shown in table 2. In the deficient group three rats failed to implant, four rats resorbed, and four rats littered. In the control group restricted in calories, only two rats failed to implant and the remainder of the rats cast litters. The restriction in calories varied from 43–84% (average 69%) of the food intake by rats given the diet ad libitum. The increased efficiency of food utilization by rats receiving pantothenic acid can be seen by comparing the weight changes during the 22-day gestation period of the deficient and caloric control rats, regardless of the occurrence of implantation.

The average number of young per litter and their average weight at birth were the same for both control groups, despite the difference in food intake. However, the rats receiving the diet ad libitum gained much more during gestation than did the rats restricted in calories.

#### *Growth and survival of suckling young rats*

The growth and survival of the deficient suckling young born under the experimental conditions are shown in table 3. The data for young deficient in pantothenic acid from birth are included for comparison. Increasing the period of the deficiency decreased both growth and survival proportionally. In every case the use of the deficient diet 846 instead of diet 841 seemed to accentuate the deficiency. This may also be

TABLE 2

*Effect of pantothenic acid deficiency and limited caloric intake on reproduction deficient diet started 16-23 days before gestation.*

EXPERIMENTAL GROUP	RAT	BODY WT. AT MAT-ING	WT. CHANGE DURING GESTATION <sup>1</sup>	AV. DAILY FOOD IN-TAKE <sup>1</sup>	RESULT OF BREEDING			
					Im-plantation	No. implan-tation sites	No. of young cast <sup>2</sup>	Av. wt. young
		gm	gm	gm				gm
Diet 841 pantothenic acid deficient	1	196	0	9.9	—	0		
	2	200	— 22	7.3	—	0		
	3	211	+ 7	10.2	—	0		
	4	223	— 5	12.8	+	8	0	
	5	235	— 34	8.6	+	7	0	
	6	244	+ 16	14.3	+	8	0	
	7	268	+ 13	12.9	+	12	0	
	8	240	+ 32	11.9	+	9	4 <sup>3</sup>	4.0 <sup>3</sup>
	9	250	+ 48	13.8	+	10	8 + 1	4.8
	10	265	+ 44	13.3	+	11	5	5.4
	11	296	+ 12	10.4	+	14	9	3.8
Diet 841 + CaP <sup>4</sup> paired feeding controls	1	186	+ 4	9.9	—	0		
	2	202	— 2	7.4	+	8	3	5.7
	3	201	+ 38	10.2	+	9	9	5.2
	4	290	+ 63	12.7	+	11	10	5.2
	5	236	— 19	8.6	—	0		
	6	240	+ 87	14.3	+	11	11	5.6
	7	270	+ 68	13.0	+	9	9	6.0
	8	242	+ 65	11.9	+	10	8	6.1
	9	255	+ 71	13.8	+	9	9	5.3
	10	259	+ 69	13.3	+	10	9	5.6
	11	280	+ 45	10.4	+	8	7	5.7
Diet 841 + CaP <sup>4</sup> ad libitum controls	1	175	+ 22	13.2	—	0		
	2	202	+ 74	13.3	+	10	9	5.9
	4	218	+ 96	17.1	+	10	9	5.9
	5	236	+ 126	20.1	+	7	7	6.1
	6	246	+ 99	17.0	+	11	0 + 3	5.0
	7	258	+ 84	17.8	+	10	10	6.3
	8	244	+ 84	16.6	+	8	5 + 2	5.4
	9	256	+ 96	18.8	+	8	8	5.8
	10	262	+ 100	18.4	+	9	9	5.8
	11	270	+ 99	16.3	+	10	10	6.2

<sup>1</sup> Data determined during the 22 days following mating.

<sup>2</sup> Living plus dead.

<sup>3</sup> These young were living foetuses found in the uterus at autopsy.

<sup>4</sup> 1 mg calcium pantothenate daily.

TABLE 3

*Effect on growth and survival of young from mothers on pantothenic acid deficient diets instituted at parturition or during pregnancy.*

EXPERIMENTAL GROUP	DIET NO.	NO. OF YOUNG	AV. WT. DAY 1	AV. WT. DAY 10	SUR- VIVAL DAY 10	AV. WT. DAY 21	SUR- VIVAL DAY 21	AV. WT. DAY 30	SUR- VIVAL DAY 30	AV. WT. DAY 60	SUR- VIVAL DAY 60	AV. LIFE-SPAN DAYS
Day of birth	841	175 ♂	6.1	16.6	95%	28	84%	31	61%	47	13%	35.4 ± 1.3 <sup>1</sup> (5-109)
		153 ♀	5.9	16.7		27		29		45		
13th day of gestation	846	40 ♂	6.3	17.9	99%	30	81%	32	33%	51	1%	28.9 ± 1.3 (5-76)
		38 ♀	6.0	16.6		27		31				
1st day of gestation	841	29 ♂	5.8	14.6	79%	19	23%		0%			15.7 ± 0.9 (5-27)
		24 ♀	5.4	14.3		21						
1st day of gestation	846	31 ♂	6.1	17.3	96%		0%					14.5 ± 0.5 (2-20)
		24 ♀	5.7	16.0								
1st day of gestation	841	17 ♂	5.4	11.0	20%		0%					6.1 ± 0.7 (1-15)
		23 ♀	4.7	14.0								
1st day of gestation	846	11 ♂	5.7		0%							4.0 ± 0.5 (1-9)
		11 ♀	6.0									

<sup>1</sup> Standard error of the mean.

noted in the data on reproductive performance (table 1). This effect may be due to improvements in the basal diet which improve growth and so increase the pantothenic acid requirement or to the presence in larger amounts of some factor accentuating pantothenic acid deficiency such as nicotinic acid (Morgan and Simms, '39) or choline (Mills et al., '40).

#### DISCUSSION

Many different dietary conditions have been reported to cause resorption in the pregnant female rat: inanition (Barry, '20), vitamin E deficiency (Evans and Bishop, '22 b), vitamin A deficiency (Sure, '28), a deficiency in the essential fatty acids (Burr and Burr, '30), a low protein intake (Guilbert and Goss, '32), vitamin B deficiency (Ueno, '34); filtrate factor deficiency,<sup>9</sup> pantothenic acid deficiency (Jukes, '40), riboflavin deficiency (Warkany and Nelson, '42), tryptophane deficiency (Albanese, Randall and Holt, '43), and very recently, biotin deficiency (Kennedy and Palmer, '45). In some of the studies reported the interpretation of results is difficult because of the concurrent effects of inanition and of one or more specific dietary deficiencies. This is particularly true in the case of the vitamin B deficiency reported by Ueno ('34). Furthermore, all early work done on inanition probably included qualitative as well as quantitative deficiencies.

In the case of pantothenic acid deficiency, the experimental data given here shows that restriction of calories to 69% (or lower in a few cases) of the normal intake did not produce resorptions. In the deficient groups of rats that resorbed, the average daily food intake averaged 12.2 gm daily or 69% of the calorie intake of controls given the diet ad libitum whereas the deficient rats that littered averaged 12.4 gm daily or 70% of the ad libitum intake, thus revealing a lack of correlation between food intake and reproductive behavior. Furthermore, the possibility of dietary deficiencies other than that of pantothenic acid as a cause of resorptions under these

<sup>9</sup> Evans and Emerson, unpublished.



conditions can be eliminated by the fact that the pantothenic acid-fed group restricted in calories did not undergo resorptions. With regard to the specific question of adequate vitamin E, the deficient rats in the last experiment received a minimum of 18 mg alpha-tocopherol by the fifth day following mating. On the usual unpurified high-fat E-low diet used for bioassays 3 mg of alpha-tocopherol will prevent resorptions in standardized E-low females (Evans et al., '36). On purified low-fat diets the requirement for vitamin E is decreased (Gottlieb et al., '43).

#### SUMMARY

Reproduction has been studied in normal adult female rats placed on purified diets deficient in pantothenic acid before or during the gestation period.

Pantothenic acid deficiency instituted on the thirteenth day of gestation did not interfere with the reproductive function. Pantothenic acid deficiency instituted 16–23 days before mating or as late as the day of mating always resulted in failure of implantation, resorption, or defective litters.

The normality of reproduction in pantothenic acid-fed controls restricted in calories eliminated the factors of inanition and of other specific dietary deficiencies as causes for these marked upsets in reproduction.

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# INFLUENCE OF DIET ON PLASMA FIBRINOGEN IN THE CHICK<sup>1</sup>

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The significance of physiological and pathological factors in the production of fibrinogen by Mammalia has been studied by a number of investigators (Foster and Whipple, '22 a, b, c, d; Schultz, Nicholes and Schaefer, '25; Vars, '30; Ham and Curtis, '38<sup>2</sup>). The studies of Whipple ('14), Meek ('12) and Foster and Whipple ('22 a, b, c, d) on varied phases of the formation of blood fibrinogen led to the important conclusion that the liver is the only potential source of fibrinogen, and more recent investigations have confirmed this view (Smith, Warner and Brinkhous, '37).

Although it was recognized at least as early as 1908 that the degree of liver injury resulting from toxic agents could be influenced by the dietary regime (Wells, '08; Davis and Whipple, '19; Miller and Whipple, '42; Neale and Winter, '38; Field, Graf and Link, '46), that dietary substances, per se, were concerned in fluctuations of blood fibrinogen was apparently first suggested by Foster and Whipple ('22 b). Their trials indicated that diets rich in animal protein, as meat,

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<sup>2</sup> The extensive literature on this subject is treated in the selected references mentioned here.

liver, etc., favored higher levels of fibrinogen in the dog than diets rich in fat and carbohydrates, or fasting, alone.<sup>3</sup> Vars ('30) reported that high protein diets act only as a transient stimulus to fibrinogen production in the dog and he concluded that the type of protein did not play any significant rôle in determining the response. In contrast to these experimental studies, Ham and Curtis ('38) did not find that fasting or food ingestion altered the pre-test fibrinogen levels in normal human subjects. However, the ingestion by humans of moderate or large amounts of animal protein produced a mild fibrinogen response. That "active substances" in animal proteins (Foster and Whipple, '22, p. 409) are involved obtains some support in the finding of Field, Sveinbjornsson and Link ('45), that certain xanthine (purine) substances are capable of elevating the plasma fibrinogen of dogs and rabbits.

In this laboratory, an earlier investigation (Field and Dam, '45) indicated that the plasma fibrinogen of chicks could readily be altered by manipulation of the diets.<sup>4</sup> These results are given here, as is a résumé and additional findings, which indicate that the fibrinogen levels of avian species are influenced by dietary means in a manner similar to Mammalia.

#### METHODS

Approximately 200 day-old White Leghorn chicks were used in these investigations. Five to ten chicks were usually placed in each experimental and control group. They were fed either of two diets, a commercial growing chick ration or synthetic ration (table 1).

Blood samples were taken at weekly intervals. Nine-tenths milliliter of blood was withdrawn by cardiac puncture without anesthesia into a syringe containing 0.1 ml of 0.1 M sodium oxalate. Control fibrinogen, plasma protein, hematocrit de-

<sup>3</sup> An historical review is given in Davis and Whipple ('19).

<sup>4</sup> In this communication we prefer to express our findings in terms of plasma fibrinogen, the precursor of the gel-protein, fibrin. It has been customary to consider fibrin and fibrinogen as similar chemical entities (protein), the significant difference being simply a physical insolubility of the fibrin.

terminations and weight measurements used as criteria indicated that the sampling procedure provoked no abnormalities.

Total plasma protein was determined by a standard colorimetric procedure (Greenberg, '29). Plasma fibrinogen was determined by a modification (Reiner, '41) of the colorimetric method of Folin and Ciocalteu ('27) as measured with a photoelectric colorimeter. However the fibrin clot obtained with chick plasma could not be manipulated with a stirring rod as is done in handling mammalian plasmas. The clot,

TABLE 1  
*Composition of basal rations.*

STOCK RATION <sup>1</sup>	SYNTHETIC RATION	gm
Corn meal	Casein (alcohol-extracted)	15
Hominy feed	Dried yeast (ether-extracted) <sup>2</sup>	10
Pulverized oats	Salt mixture <sup>3</sup>	7.2
Pulverized barley	Gelatin	8
Wheat bran and middlings	Gum arabic	5
Soy bean oil meal	Choline chloride	0.1
Alfalfa meal	l-cystine	0.1
Meat scraps	Sucrose	54.6
Bone meal	Vitamin K substitute <sup>4</sup>	.001
Gluten meal	Vitamin A, D concentrate <sup>5</sup>	
Distiller's grain with solubles	1 drop twice weekly	
Dried skim milk and buttermilk		
Dried whey		
Limestone		
Salt plus Mn		
Fortified fish liver oil		
Wheat germ oil		

<sup>1</sup> Marketed as "Mystic Starting and Growing Mash" by Newman Brothers Grain Co., Rochester. The exact composition of the diet was refused. Guaranteed analysis: crude protein, not less than 20%; crude fat, not less than 3.5%; crude fiber, not more than 7%.

<sup>2</sup> Fleischmann type 2019.

<sup>3</sup> McCollum's salt mixture no. 185 7.16 gm, KI 0.966 mg, CuSO<sub>4</sub> · 5 H<sub>2</sub>O 9.93 mg, MnSO<sub>4</sub> · 4 H<sub>2</sub>O, 39.8 mg.

<sup>4</sup> Tetrasodium salt of 2-methyl-1,4-naphthohydroquinone diphosphoric acid (Synkayvite-Roche).

<sup>5</sup> Consists of: vitamin A concentrate from fish liver oil (containing 10<sup>6</sup> units per gm) 0.300 gm, vitamin D concentrate (Delsterol, containing 200,000 chick units per gm) 0.200 gm, oleic acid 24,500 gm. The concentrates were obtained from Distillation Products, Inc., Rochester. One drop (29 mg) represents 350 units A and 46 chick units D.

developed in a 15 ml centrifuge tube, was broken into small threads and fragments by vigorous stirring with the rod, centrifuged down to a mat, and both tube and clot were freed of the plasma protein in the supernatant layer by repeated washing with distilled water. The fibrin was hydrolyzed, and the color developed in a standard manner (Reiner, '41).

*Fibrinogen content of plasma from chicks  
'with avitaminosis K*

The need for further modification in the procedure for measuring plasma fibrinogen of chicks became evident from a study of the fibrinogen level of chicks raised on a vitamin K-free diet. The fibrin clot obtained from these chicks was developed with difficulty and gave abnormally low values. However, this tendency was not a characteristic of the hypoprothrombinemia of a vitamin K deficiency. The plasma fibrinogen from several chicks receiving the same synthetic diet supplemented with adequate quantities of vitamin K was similarly depressed. The addition of small amounts of bovine thrombin<sup>5</sup> served to restore normal coagulation and give optimum fibrinogen values. Variations in the quantity of thrombin added did not measurably affect the fibrinogen values (Seegers, Niefert and Loomis, '45). The standard procedure adopted consisted of adding approximately 0.5 mg thrombin (approximately 15 Iowa units in solution with calcium chloride) to 0.4 ml oxalated plasma diluted to 10 ml with saline solution.

In several trials, duplicate determinations of the plasma fibrinogen were performed using the colorimetric procedure and a gravimetric method (Foster and Whipple, '22 a). The values obtained with both methods were in agreement.

EXPERIMENTAL

*Effects of basal diets on fibrinogen levels*

In the initial tests, a synthetic diet was used which differed only slightly from that given in table 1 (alcohol-extracted

<sup>5</sup> Parke-Davis.

casein 15 gm, ether-extracted brewer's yeast 7.5, gelatin 8, salt mixture 5, gum arabic 5, cystine 0.1, cod liver oil 5, d,l-alpha tocopherol acetate 0.005, and corn starch 54.4). This basal diet was supplemented with either 0.5 gm choline chloride, at the expense of the corn starch, or 0.003 gm of a vitamin K compound, the tetrasodium salt of 2-methyl-1,4-naphthohydroquinone diphosphoric acid.<sup>6</sup> The fibrinogen levels of 4-week old chicks receiving the basal diet noted above, supplemented with either vitamin K or choline, and chicks receiving the natural ration, are given in table 2. High

TABLE 2  
*Plasma fibrinogen levels of 4-week-old chicks.<sup>1</sup>*

DIET	AVERAGE AND S.D. <sup>2</sup> mg %	RANGE mg %
Basal synthetic diet <sup>3</sup>	423 ± 133	285-594
Basal plus vitamin K <sup>4</sup>	435 ± 96	319-602
Basal plus choline <sup>5</sup>	505 ± 44	385-590
Control natural diet	245 ± 66	171-335

<sup>1</sup> Average of 9 chicks in each group.

<sup>2</sup> Standard deviation.

<sup>3</sup> Free of vitamin K and low in choline (see text).

<sup>4</sup> Synkayvite-Roche (tetrasodium salt of 2-methyl-1,4-naphthohydroquinone diphosphoric acid) 30 mg per kg ration.

<sup>5</sup> Choline ehloride, 5 gm per kg ration.

fibrinogen values were common to the plasma of all chicks raised on the basal synthetic diet irrespective of its choline or vitamin K content, whereas the fibrinogen in chicks raised on the natural ration was relatively low. The growth of the chicks receiving the natural ration was somewhat greater than those given the synthetic diet. After 4 weeks, the average weights were as follows: basal synthetic diet 143 gm, plus vitamin K 128 gm, plus choline 166 gm, natural ration 208 gm. The plasma prothrombin time of the chicks given either the natural ration or the synthetic diet supplemented with vitamin K was within the normal range, whereas the prothrombin time of the chicks given the unsupplemented or choline-supplemented synthetic diet was drastically prolonged (Field and Dam, '45).

<sup>6</sup> Synkayvite-Roche.



*Interchanging rations of chicks*

Two groups of chicks were fed either the basal synthetic or the natural ration (table 1) for 4 weeks. The fibrinogen levels at this time were characteristically elevated in the chicks receiving the synthetic ration, and relatively low in the chicks receiving the natural ration (table 3). When the animals were shifted from one diet to another the capacity of the synthetic ration to increase fibrinogen values appeared within 4 days while at the end of 1 week the fibrinogen levels had increased

TABLE 3

*Plasma fibrinogen levels of chicks, raised on the basal synthetic and natural rations, when diets were interchanged.<sup>1</sup>*

WEEKS ON RATION	GROUP 1			GROUP 2		
	Plasma fibrinogen		Range	Plasma fibrinogen		Range
	Average			Average		
	mg %	mg %		mg %	mg %	
4 <sup>2</sup>	437	334-509	(Synthetic ration)	187	152-202	(Natural ration)
1	301	258-360	(Natural ration)	550	397-699	(Synthetic ration)
2	332	279-395	(Natural ration)	427	359-495	(Synthetic ration)
3	263	194-365	(Natural ration)	391	345-436	(Synthetic ration)

<sup>1</sup> Average of 5 chicks in each group.

<sup>2</sup> The chicks were 4 weeks old at the time the diets were exchanged.

to 300% over the levels which the chicks maintained on the natural ration. The precipitous increase leveled off and readjustment to slightly lower values occurred in subsequent weeks (table 3). Chicks changed from the synthetic ration to the natural ration underwent a less abrupt change. The initially elevated fibrinogen values were reduced after the chicks had consumed the natural ration for 1 week, and the decrease progressed during subsequent weeks.

*Effect of high vitamin K intake*

The fibrinogen levels of chicks reared on diets free of vitamin K or supplemented with minimum quantities of the naphthoquinone sufficient to prevent a hypoprothrombinemia (Dam, '42) were similar (table 2). Palladin ('45) has stated that vitamin K<sub>3</sub> plays a rôle in the fabrication of fibrinogen, while Field and Link ('45) found that large single oral doses of various forms of vitamin K not only induce a state of temporary hyperprothrombinemia but may also temporarily elevate the fibrinogen levels in some rabbits.<sup>7</sup> The effect of a continued high vitamin K intake in chicks was tested by adding 0.03 gm of a vitamin K-substance<sup>8</sup> to a kilo of the natural (vitamin K-containing) ration given 3-week old chicks. The fibrinogen levels of the chicks were increased after they had consumed the supplemented ration for 14 days (starting plasma level 289 mg %, after 14 days 365 mg %), but thereafter fell to the pre-test normal values. Increasing the added vitamin K to 0.06 gm per kilo of ration had no further influence on the plasma fibrinogen levels.

*Effect of different animal proteins*

By simultaneously replacing 15% of the casein and 15% of the sucrose in the synthetic ration (table 1), the following animal protein substances were tested at a dietary level of 30%: whole dried hog liver,<sup>8</sup> and whole dried beef liver.<sup>8</sup> In addition, one group was given a diet containing 30% casein, and another group of chicks was placed on a diet in which the protein consisted of 15% casein plus 15% whole dried beef liver. The effect of these diets on the growth, total plasma protein and plasma protein is presented in table 4. The chicks given the ration containing the hog liver did not survive more than 4 weeks, prior to which the plasma protein and fibrinogen were markedly decreased. Although chicks given the diets containing either beef liver or casein (30%) exhibited the

<sup>7</sup> Field, J. B., and K. P. Link, unpublished experiments.

<sup>8</sup> Armour.

same plasma protein values, they differed in their plasma fibrinogen content. The fibrinogen levels of chicks given the 30% casein diet remained relatively low while the plasma fibrinogen of chicks given the beef liver diet increased to levels approximating those obtained when the 15% casein diet was fed (tables 2 and 3). The protein content of the regular synthetic diet (15% casein) was also increased by replacing 15% sucrose with beef liver. However, this diet did not prevent some increase in plasma fibrinogen above the level attained with the 30% casein diet (table 4).

TABLE 4

*Effect of synthetic diets containing different proteins on the average growth, total plasma protein and plasma fibrinogen of chicks.<sup>1</sup>*

AGE WEEKS	30% HOG LIVER			30% BEEF LIVER			30% CASEIN			15% CASEIN — 15% BEEF LIVER		
	Weight (gm)	Total plasma protein (gm %)	Fibrinogen + S.D. <sup>2</sup> (mg %)	Weight (gm)	Total plasma protein (gm %)	Fibrinogen + S.D. (mg %)	Weight (gm)	Total plasma protein (gm %)	Fibrinogen + S.D. (mg %)	Weight (gm)	Total plasma protein (gm %)	Fibrinogen + S.D. (mg %)
1	59.3			64.2			60.9			65.4		
2	72.8		215	78.7		263	88.9		170	96.7		218
		± 29			± 54			± 38			± 26	
3	101.9	2.9	180	135.2	3.3	362	120.6	3.5	175	155.6	3.9	275
		± 38			± 117			± 48			± 41	
4	128.0	1.6	169	195.4	3.1	308	171.9	3.3	224	214.7	3.4	344
		± 27			± 31			± 32			± 21	
5	Dead			238.7	3.4	342	211.3	3.5	217	280.7	3.6	338
					± 20			± 97			± 37	
6				286.0	3.4	354	246.3	3.6	219	337.1	3.7	295
					± 39			± 44			± 43	

<sup>1</sup> Ten chicks in each group.

<sup>2</sup> Standard deviation.

### *Effect of low protein and low casein diets*

A group of ten chicks was given the regular synthetic ration until they attained 3 weeks of age. At this time, 5 of the chicks were transferred to the same basal ration in which the casein was withdrawn and replaced by sucrose. Thus the

only possible source of protein in this new diet was the yeast and 8% gelatin. Subsequently, the growth of the chicks consuming the casein-free ration ceased (table 5) and gross symptoms of dietary deficiency developed (loss of feathers, stunting of growth, etc.). The plasma protein of these chicks was decreased between the third and sixth weeks on this regime, but the fibrinogen level fell only slightly. During this period the plasma fibrinogen of the control chicks receiving

TABLE 5

*Effect of synthetic diets, with and without casein, on the average growth, total plasma protein and fibrinogen of 3-week-old chicks.<sup>1</sup>*

AGE WEEKS <sup>3</sup>	TIME ON DIET TESTED WEEKS	15% CASEIN			NO CASEIN <sup>2</sup>		
		Weight gm	Total plasma protein gm %	Fibrinogen mg %	Weight gm	Total plasma protein gm %	Fibrinogen mg %
3	1	153	3.3	286	133	3.0	274
4	2	193	3.3	324	126	3.0	275
5	3	242	3.3	329	121	2.6	261
7	5	383	3.2	386	116	2.8	256
9	7	443	3.2	370	127	2.9	267

<sup>1</sup> Five chicks in each group.

<sup>2</sup> The casein withdrawn from the diet was replaced by sucrose. A small amount of protein in the remaining formula was provided by gelatin and the yeast.

<sup>3</sup> All chicks were raised from hatching until 3 weeks of age on the regular synthetic ration.

the 15% casein ration was somewhat increased (table 5). When a similar ration containing only 5% casein was given to another group of 1-day old chicks, the fibrinogen levels were, by comparison with chicks of the same age receiving other diets, strikingly elevated within 1 week (table 6). Blood samples withdrawn at weekly intervals thereafter revealed a reduction in total plasma proteins while the average plasma fibrinogen levels were elevated.

### *Effect of fasting*

The stimulating effects of diet on the fibrinogen levels in dogs can be removed by the withdrawal of food for a period

of approximately 5 days (Foster and Whipple, '22 b; Vars, '30). This procedure reduced the plasma fibrinogen to a stable low uniform level. One group of 8-week-old chicks raised on the synthetic diet was fasted for 5 days during which it had access only to water. As a result of fasting the following changes occurred: the body weight was reduced to 332 gm from 443 gm; total plasma proteins were reduced to 2.6 gm % from 3.5 gm %; and plasma fibrinogen was reduced to 222 mg % from 286 mg %.

TABLE 6

*Effect of different diets on the average growth, total plasma protein and fibrinogen of chicks.<sup>1</sup>*

AGE WEEKS	NATURAL RATION			SUPPLEMENTED NATURAL RATION <sup>2</sup>			SYNTHETIC DIET PLUS VITAMIN E <sup>3</sup>			SYNTHETIC DIET MINUS VITAMIN E			SYNTHETIC DIET LOW CASEIN <sup>4</sup>		
	Weight (gm)	Total plasma protein (gm %)	Fibrinogen (mg %)	Weight (gm)	Total plasma protein (gm %)	Fibrinogen (mg %)	Weight (gm)	Total plasma protein (gm %)	Fibrinogen (mg %)	Weight (gm)	Total plasma protein (gm %)	Fibrinogen (mg %)	Weight (gm)	Total plasma protein (gm %)	Fibrinogen (mg %)
1	65	2.9	222	65	3.5	215	55	3.0	221	54	—	—	41	3.2	317
2	113	3.1	228	118	3.4	211	85	3.2	283	84	3.3	228	44	2.8	307
3	185	3.0	222	192	3.1	233	117	3.3	405	110	3.1	349	51	2.4	277
4	260	3.0	222	252	3.1	265	142	3.3	455	124	3.4	414	55	2.8	429

<sup>1</sup> Five chicks in each group.

<sup>2</sup> Natural ration plus 3% liver fraction L and 5% yeast.

<sup>3</sup> 10 mg d,l-alpha tocopherol acetate (Ephynal-Roche) per 100 gm diet.

<sup>4</sup> Containing only 5% casein, sucrose replacing the 10% casein withdrawn from the regular synthetic diet.

### *Effect of miscellaneous rations*

In table 6 are presented data obtained from chicks given varied diets; the natural ration, the natural ration to which was added both 5% yeast and 3% liver fraction L,<sup>2</sup> and the synthetic ration with and without vitamin E. The supplemented natural ration gave results very similar to the natural

<sup>2</sup> Wilson Laboratories.

ration alone. The protein levels obtained in chicks receiving the synthetic ration with or without vitamin E were essentially the same. In further trials, liver fraction L at a level of 3% was incorporated into the synthetic ration consumed by 8-week-old chicks, and brewer's yeast, at a 5% level was added to the natural ration consumed by a like group of chicks. The fibrinogen level of the chicks consuming the supplemented synthetic ration remained elevated, that of the chicks receiving the natural ration was not increased by the added yeast.

#### DISCUSSION

The facility with which levels of fibrinogen, a plasma globulin, can be influenced by the dietary regime is greater than has been generally recognized. By contrast, the serum globulins are relatively inert to the effects of the diet. It may be of significance that the latter probably originates from several organs or tissues while fibrinogen production is most likely an exclusive function of the liver (Madden and Whipple, '40). Although the level of plasma prothrombin in some species readily responds to diverse stimuli and influences (Link, '43-'44) the prothrombin levels of the chicks studied in similar trials (Field and Dam, '45) do not indicate that detectable differences in prothrombin levels can be induced in normal chicks (supplied with adequate amounts of vitamin K) even by drastic alterations in the dietary intake.

In a recent report, Zeldis et al. ('45) record observations of a three-fold increase in the plasma fibrinogen of a dog maintained on a low protein diet for a prolonged period. Similarly, the present studies with chicks indicate that the level of plasma fibrinogen directly reflect the nature and relative quantity of the dietary protein. A basal synthetic ration with 15% casein such as that employed in this study has found extensive use in the past and yet the chicks raised on this ration evidence high fibrinogen levels. Casein-free (low-protein) or low-casein low-protein diets resulted in some elevation of fibrinogen levels, while lower fibrinogen levels are characteristic of chicks consuming the same ration containing

30% of casein, or a natural ration containing approximately 20% mixed proteins. Thus, it is proposed that high fibrinogen levels in chicks may be a manifestation of an inadequate protein intake. Since fasting alone reduced the plasma fibrinogen to levels attained on the high casein and natural rations it would appear that a stimulus to fibrinogen production stems from consumption of diets in which the protein is inadequate, qualitatively or quantitatively. Prolonged consumption of protein-poor diets has been shown to produce liver cirrhosis in some species, and one ready manifestation of liver injury is an elevation of the plasma fibrinogen levels (Foster and Whipple, '22 b). In the rapidly growing chick, a light degree of protein inadequacy might be rapidly reflected in a hepatic fibrinogenic response. Older chicks can be made to show an elevation in fibrinogen levels less readily than young chicks when given the 15% casein diet. The high requirement of the growing chick for certain amino acids such as methionine, glycine, arginine (Hegsted et al., '41) suggests that the effect of supplementing this diet with selected amino acids, or combinations of these, on the elevated fibrinogen levels bears further investigation. Full rationalization of the response and its importance in the economy of the animal is not yet at hand.

The present data do not support the view that animal proteins, per se, stimulate the production of plasma fibrinogen (Foster and Whipple, '22 b; Vars, '30). A high and adequate casein consumption did not stimulate fibrinogen production in the chick. Thus it is more plausible that certain protein-rich materials contain substances (purines (?) Field, Sveinbjornsson and Link, '45), which are capable of inducing this effect. Whole dried hog liver in contrast to whole dried beef liver (incorporated into a synthetic diet at a level of 30%), resulted in relatively low plasma fibrinogen levels (concurrent with falling total plasma protein). However, since hog liver is toxic to vitamin E-deficient chicks (Dam, '44), and was incapable of supporting chicks in the present trials, it appears likely that lower fibrinogen levels obtained on diets containing this tissue may be entirely attributable to a toxic action.

## CONCLUSIONS

1. The plasma fibrinogen levels of growing chicks were influenced by the dietary regime. Synthetic diets containing 15% casein produced high fibrinogen levels while the consumption of a diet containing 30% of casein or a natural grain ration (20% protein) gave relatively low fibrinogen values. These changes in plasma fibrinogen were independent of the growth, and the level of total protein in the plasma of the chicks.

2. The total plasma protein of chicks consuming synthetic diets low in casein (protein deficient) or a casein-free diet, was reduced while the plasma fibrinogen was elevated. Fasting reduced elevated fibrinogen levels to the low levels characteristic of the adequate natural ration and lowered the total plasma protein.

3. When chicks consumed a natural ration to which was added relatively huge amounts of vitamin K, a temporary slight increase in plasma fibrinogen was obtained and this state persisted for 14 days.

4. While a synthetic diet containing 30% dried whole hog liver resulted in reduced total plasma protein and low fibrinogen levels, and eventually death, 30% whole dried beef liver induced elevated fibrinogen levels. A diet containing 15% casein and 15% whole dried beef liver failed to prevent the elevation in plasma fibrinogen obtained with the 15% casein diet.

5. The following substances did not influence the fibrinogen levels of chicks when they were added to either a basal synthetic diet or a natural ration: choline, brewer's yeast, liver fraction L, or alpha-tocopherol acetate.

6. It is suggested that the elevated fibrinogen levels reflect a metabolic disturbance of the liver in utilizing a dietary protein intake which is inadequate, qualitatively or quantitatively.



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# OBSERVATIONS ON EFFICIENCY OF SWIMMERS AS RELATED TO SOME CHANGES IN PRE-EXERCISE NUTRIMENT<sup>1</sup>

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Physical performance in relation to diet has been the subject of fairly extensive investigation (for review of the literature see Keyes, '43). Muscular efficiency as related to the immediate antecedent food intake has also been studied (Carpenter and Fox, '31; Haggard and Greenberg, '35; Haldi, Bachmann, Ensor and Wynn, '38), but the effect of various foods and the amount of food eaten shortly before strenuous exercise of short duration has received only scant attention. Among athletic coaches and trainers, who might reasonably be expected to have learned from experience and observation whether athletic performance is affected by the type of meal eaten before a contest, there is a wide diversity of opinion on this subject. It has been shown recently (Haldi and Wynn, '45) that in brief exhausting exercise, work output and the onset of fatigue are not affected by the kind of food consumed 2 to 3 hours before exercise. The present experiments were undertaken for the purpose of making a similar study on the effect of variations in the caloric intake several hours before exercise.

## PROCEDURE

Swimming a hundred yard sprint is well adapted for studying any changes that might occur in the capacity for work dur-

<sup>1</sup> The expense of this investigation was defrayed by a grant-in-aid from the Sugar Research Foundation.

ing strenuous exercise of short duration. The metabolic rate is raised 10 to 50 times above the basal (Karpovich and Millman, '44) and although the exercise is exhausting, it is relatively easy to provide sufficient incentive for the swimmer to make a maximum exertion throughout the exercise period. Comparable quantitative criteria of the capacity for work under different experimental conditions are afforded by the time required to swim three laps, each being one-third the total distance. The drop-off in the second and third laps as compared with the first may be taken as an objective criterion of fatigue in the course of exercise.

Twelve subjects who volunteered to take part in the experiment were selected by the swimming coach from a group of healthy young men in the Navy V-12 training unit at Emory University. All had engaged in daily compulsory swimming for some time but they were nevertheless subjected to further intensive training for the experiment. Although there was practically no improvement in their performance from day to day, they were none-the-less eager to improve and readily responded to the urging that was given them before each experiment to try for a new record. The V-12 training program provided a highly desirable uniformity of living conditions. There were some variations in the usual daily activities over the week-end and on this account the experiments were conducted only on Tuesdays and Thursdays.

On the days of the experiments either a specially prepared light or heavy meal was served in the mess hall at 4:30 P.M. The composition of the meals is presented in table 1. To study the effect of additional nutriment in the form of readily absorbable carbohydrate the light meal was supplemented on certain days by 50 gm of cane sugar 1 hour before swimming and on other days by 100 gm of sugar. The order of the first experiments was as follows: 1, heavy meal; 2, light meal; 3, light meal plus 50 gm of sugar; 4, light meal plus 100 gm of sugar. These four different experiments will be referred to as a set of experiments. In each set the order of the preceding set was reversed to offset any error that might be intro-

duced if there should be a gradual improvement in the performance as a result of the repetitious efforts of the swimmers to make a new record.

On the day of the experiment the subject reported at the pool at 7:00 P.M. This hour of the day was chosen so as not to interfere with the trainee's classwork and other assign-

TABLE 1  
*Composition of "heavy" and "light" meals.*

FOOD	SERV- ING	CHO	PRO- TEIN	FAT	CAL.	SERV- ING	CHO	PRO- TEIN	FAT	CAL.
		gm	gm	gm			gm	gm	gm	
HEAVY MEAL						LIGHT MEAL				
Steak	10 oz.	0	82	26	562	6 oz.	0	49	14	322
Bread	1 slice	13	2	0	60	1 slice	13	2	0	60
Butter	14 gm	0	0	12	108	7 gm	0	0	6	54
Egg (hard boiled)	1	0	7	5	73	$\frac{1}{2}$	0	4	3	43
Green peas	4 oz.	11	4	0	60	2 oz.	6	2	0	32
Tomato	1 slice	1	0	0	4	1 slice	1	0	0	4
Mayon- naise (on tomato)	2 soup spoons (level)	0	1	16	148					
Peaches (canned)	1 half	8	1	0	36	1 half	8	1	0	36
Total		33	97	59	1051		28	58	23	551
Total cal.		132	388	531	1051		112	232	207	551
% of total caloric intake		13	37	50	100		20	42	38	100

ments. A blood sample was obtained by a finger puncture and the subject warmed up by swimming slowly 100 yards. The warming up exercise was followed by a brief rest period at the conclusion of which the subject toed the mark as in the usual swimming meet and at a given signal dove into the

pool. While swimming he was continuously exhorted to swim faster. Each of the laps of  $33\frac{1}{4}$  yards was timed in split seconds. At the conclusion of the swim the subject was given 3 to 5 minutes to regain his wind and then another finger puncture was made. The blood samples drawn before and after the swim were analyzed for sugar by the Hagedorn-Jensen procedure (Peters and Van Slyke, '32). The purpose of obtaining the blood samples was to determine whether there was any correlation between the blood sugar level and the food intake before exercise. In the event of such a correlation it would be of further interest to ascertain whether there was any relationship between the blood sugar level and swimming performance.

### RESULTS

The swimming time and the drop-off in the second and third lap as compared with the first was the same regardless of the antecedent nutriment. The experimental data are presented in table 2. The blood sugar concentration was also the same

TABLE 2

*Swimming time and blood sugar concentration 2½ to 3 hours after a heavy meal, a light meal, and a light meal when sucrose was ingested 1 hour before swimming.<sup>1</sup>*

NUTRIMENT BEFORE SWIMMING	TIME				DROP-OFF		BLOOD SUGAR	
	1st $33\frac{1}{4}$ yards	2nd $33\frac{1}{4}$ yards	3rd $33\frac{1}{4}$ yards	100 yards	2-1 $33\frac{1}{4}$ yards	3-1 $33\frac{1}{4}$ yards	Before swim	After swim
	<i>seconds</i>	<i>seconds</i>	<i>seconds</i>	<i>seconds</i>	<i>seconds</i>	<i>seconds</i>	<i>mg %</i>	<i>mg %</i>
Heavy meal	20.5	25.8	29.1	75.4	5.4	8.6	126	148
Light meal	20.6	25.8	29.5	75.9	5.3	8.9	121	142
Light meal + 50 gm sucrose	20.6	25.9	29.3	75.8	5.3	8.7	128	139
Light meal + 100 gm sucrose	20.7	25.7	29.3	75.7	5.1	8.6	124	141

<sup>1</sup> Each value in the table is an average of 53 experiments except the blood sugar concentrations which are averages of 45 blood samples.

before swimming in the different experiments. There was invariably a rise in the blood sugar after swimming, but the extent of the rise was the same on the various food intakes.

All the averages in the table on the swimming time are derived from 53 experiments. It was our original intention to do 5 experiments on 12 subjects after each type of feeding which would have given 60 determinations on each desired datum. On a few occasions, however, a subject was unable to swim in the scheduled experiment. Absence from one experiment automatically disqualified him from taking part in the other 3 experiments of the set. The averages in the tables are therefore strictly comparable as they were all obtained from the same number of experiments and on the same subjects. Eight blood samples were lost, and in accordance with the rules of our procedure the samples on the other 3 experiments in the set were discarded. Accordingly each of the averages on blood sugar concentration was obtained from 45 experiments.

#### DISCUSSION

It is a reasonable assumption that the energy reserves of a well developed and properly nourished individual are adequate for strenuous exercise of short duration. There are some, however, who believe on a purely empirical basis that even in short bouts of exercise one's capacity for work is related to the amount of food eaten before exercise. One short distance swimmer who had been a successful contestant in the Olympics, for example, informed one of us (J.H.) that he always ingested some dextrose an hour or so before an event. His "experience" had convinced him that the extra energy supplied by the sugar enabled him to swim faster and with less fatigue. Similar opinions have been expressed by various athletic coaches. There are those on the other hand who have the definite impression that they perform best in athletic exercise after a light meal (Hellebrandt and Karpovich, '41). This point of view is probably based on the assumption that after a heavy meal a distended stomach may interfere with



the descent of the diaphragm or that digestive and other processes might interfere with the physiological adjustments that take place in exercise. These conflicting empirical deductions suggested the advisability of obtaining precise information by experimental studies.

The present experiments show that performance in severe exercise of brief duration is not affected by the amount of nutriment taken 2 to 3 hours before exercise. In previous experiments it has been found that swimming performance is not affected by the kind of food eaten beforehand (Haldi and Wynn, '45). It need not be emphasized that without further experimentation these conclusions can not be regarded as applicable to long protracted and exhausting exercise.

The decrease in speed which may be quantitated as the drop-off in the second and third laps as compared with the first serves as an objective criterion of fatigue. Obviously, fatigue during swimming, as measured by this standard is not related to the amount of food taken before swimming nor to a fall in the blood sugar while swimming, since the blood sugar concentration was higher immediately after than before exercise. It is worthy of note that the blood sugar level before swimming was the same when 50 or 100 gm of sucrose had been ingested approximately 1 hour after the meal as when no sugar was taken. This may have been due to the same mechanism which prevents a rise in blood sugar when the second ingestion of glucose follows the first within an hour and a half (Foster, '23).

A rise in the blood sugar concentration after severe exercise has been observed also by other investigators. From observations on football players, the conclusion has been offered that hyperglycemia is uncommon in exercise with little or no emotional stress but common in exercise with emotional stress on the football field (Edwards, Richards and Dill, '31). This point of view, however, does not appear to have been substantiated by later experiments (Dill, Edwards and Mead, '35). Blood sugar concentration was found to be increased 10 to 66% when the subjects performed work that brought on

exhaustion within 10 to 40 minutes. The type of work done was of a nature that should not have been conducive to emotional stress. It appears likely that the high blood sugar level immediately after swimming in our experiments may have been a direct effect of exercise. The subjects did not experience the emotional states that are usually aroused by competitive games and a cheering crowd. As far as could be ascertained the determined effort of the swimmer to excel his previous record did not cause any marked emotional excitement.

#### SUMMARY AND CONCLUSIONS

A study of the performance of swimmers in a hundred yard sprint has been made to determine the influence of the size of the antecedent meal on severe exercise of short duration.

Two and a half to 3 hours after a heavy meal, the time required to swim each of three laps in the hundred yard sprint was the same as when a light meal was taken.

Supplementation of the light meal by the ingestion of 50 or 100 gm of sucrose before swimming had no effect on the swimming time.

The drop-off in the second and third laps which is taken as an objective index of fatigue was the same regardless of the amount of the food intake before swimming.

The blood sugar level before swimming was the same 2 to 3 hours after the various amounts of antecedent food intake. Immediately after swimming there was a rise in the blood sugar which was practically the same in all experiments. This rise in blood sugar was apparently a direct effect of exercise and not due to emotional stress.

It is concluded that there is no relationship of speed, power and skill in swimming a hundred yard sprint to the amount of nutriment taken 2 to 3 hours before swimming.

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# BIOLOGICAL VALUE OF PROTEINS IN RELATION TO THE ESSENTIAL AMINO ACIDS WHICH THEY CONTAIN<sup>1</sup>

## I. THE ENDOGENOUS NITROGEN OF MAN

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TWO FIGURES

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The biological value method introduced by Thomas ('09) has undergone many improvements and modifications in its various applications. As Mitchell has made clear in his many articles describing improvements for application to rats and farm animals and summarized as to the fundamental principles involved in several searching reviews ('26, '42 and '44), it is not, strictly speaking a method for measurement of the biological value of a protein for maintenance except when applied to, and based on, the endogenous nitrogen excretions of adult animals.

In consideration of physiological differences between man and lower animals with respect to his tolerance of foods, this laboratory developed the use of egg protein metabolism as a relative basis, in contrast with the endogenous or absolute basis (Sumner, Pierce and Murlin, '38; Murlin, Nasset and

<sup>1</sup> The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Rochester.

Marsh, '38; Murlin, Marshall and Kochakian, '41). This modification was perfectly justified also by the high absolute biological value of this protein.

In undertaking the present studies on human subjects, we visualized an opportunity for so ordering the sequence of periods of observation as to permit the development of conversion factors which would relate values referred to endogenous excretion with those referred to egg, or any other high value food protein as base. The second paper will be concerned with this question of the interconvertibility of biological values.

The employment of the endogenous base presented other advantages aside from that of convertibility; viz., economy in the use of the very expensive synthetic amino acids; and the comparison of our results with those obtained by the endogenous method, principally on rats, in other laboratories.

A long series of studies on the same subjects was hoped for, involving perhaps the necessity of many no-protein periods; and it was feared these hopes could not be realized without encountering the risk of serious depletion of reserve protein, even to the point of liver damage, unless a reliable measure of endogenous nitrogen excretion could be accomplished in relatively short periods. It was necessary, therefore, to give consideration to the factors affecting the attainment of reliable endogenous values in the adult man.

#### BASAL DIET AND SEQUENCE OF PROTEINS

Table 1 exhibits the items of a diet in which egg protein furnished 95% of the nitrogen after deducting the nitrogen of coffee, tea and the caffeine in a bottle of carbonated beverage. As a rule the total energy value of the diet at the beginning of a series was placed at 45 cal. per kilo of net body weight, but when in individual cases it became apparent that this allowance was not sustaining body weight it was raised to 48 cal. per kilo or sometimes higher, by addition principally of carbohydrate. Even with all the carbohydrate the subjects could tolerate, however, some loss of weight could not always

be prevented. It was always heaviest in periods when amino acids were being fed.

Arrowroot starch was definitely superior to either corn starch or wheat starch as the principal ingredient of the biscuit or muffin because of its lower nitrogen content and better acceptability.

TABLE 1

*Illustrative diet, used by F. L., weighing 66 kg. At the beginning this diet contained 4.75 gm N, 378 gm carbohydrate, 149 gm fat, and furnished 2970 Cal.; later, when the Cal. were raised to 3168 (48/kg), it contained 404 gm carbohydrate and 159 gm fat.*

COMPONENT	AMOUNT	N	FAT	CAR- BO- HY- DRATE	VITAMINS PER DAY	
	gm	gm	gm	gm		
Egg	228	4.55	24		A (Navitol)	4,000 I.U.
Butter	40	0.009	40		D (Navitol)	500 I.U.
French dressing	30	0.006	23		Ascorbic acid	100 mg
Lettuce	80	0.077		3	Thiamine HCl	1.0 mg
Sugar	15			15	Riboflavin	0.8 mg
Marmalade					Niacin	10.0 mg
Biscuit (arrow-root starch)	(4 pieces)	0.084	55	148	Pyridoxine	0.5 mg
Orangeade (artificial)	408	0.0088		102	Ca pantothenate	2.0 mg
Candy	80			80	acid	0.8 mg
Coffee (2 cups)	360	0.1836			Choline HCl	1.5 mg
Tea (1 cup)	180	0.144			Inositol	100.0 mg
Cola drink	180	0.006		18		
Omelet oil	17		17			
Applesauce	100	0.0193		38		
Vitamins		0.0126				
Mineral mixture <sup>1</sup>		0.0002				
Total		5.101	159	404		
Deducting for N in coffee, tea and cola		— 0.334				
		4.767				

<sup>1</sup> The mineral mixture had the following composition in gm (for 300 man-days): CaCO<sub>3</sub>, 600; MgCO<sub>3</sub>, 78; MgSO<sub>4</sub>, 30; NaCl, 900; KCl, 345; KH<sub>2</sub>PO<sub>4</sub>, 627; FePO<sub>4</sub> · 4H<sub>2</sub>O, 20; KI, 0.08; MnSO<sub>4</sub>, 0.35; NaF, 1.00; Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub> · K<sub>2</sub>SO<sub>4</sub>, 0.17; CuSO<sub>4</sub>, 0.90. (Partly from Mattill and Mattill, *Endocrinology and Metabolism*, '23, and partly from Hubbell, Mendel and Wakeman, *J. Nutrition*, vol. 14, p. 273, '37.)

The typical sequence of periods which prevailed in more than half the series listed in table 2 started with egg protein, the distribution of calories being set at 4% for protein, 51% for carbohydrate and 45% for fat. When endogenous nitrogen was to be determined the "no-protein," consisting of the same diet except egg, followed immediately after the egg period, adjustment of calories for loss of the protein being made. Then came the test protein fed at some definite level with reference to the endogenous N, and following this came the amino acid mixture compounded in a definite relationship to the composition of the test protein. A new cycle beginning with egg protein then was started.

The subjects of these experiments were principally conscientious objectors assigned to the project by Selective Service from the quota allocated to O.S.R.D. and finally numbering 21 men and one parolee. Sixteen other men and 10 women, volunteers from the Medical School and Strong Memorial Hospital personnel, served exclusively in series 1 and 2, and in small numbers in series 3, 4 and 7.

All but one of the subjects were in apparently good health when entering upon the experiments and, not counting minor ailments, all but three continued to the end in the series to which assigned without detriment to health. The three exceptions are mentioned in connection with the discussion in this paper of the series to which they belonged.

Continuous determinations of nitrogen by the macro-Kjeldahl method were made in all principal items of the diet and in all new lots of even minor items as well as in every day's 24-hour urine and period feces. Creatinine N was regularly determined as a check on collection of urines, and amino acid N was run in the urines of several periods in which they were fed.

#### FACTORS AFFECTING THE ENDOGENOUS LEVEL OF EXCRETION

##### *(1) The starting level*

Folin ('05), in changing four subjects from a general mixed diet containing from 14 to 16.8 gm of N to his starch-cream

diet containing less than 1 gm of N, found that the lowest level of uric acid N was reached on the fourth, fifth, first and third day, respectively, for his four subjects; the lowest urea nitrogen was reached on the seventh, fourth, fifth and fifth day, respectively, and the lowest total N on the tenth, seventh, fifth and fifth, respectively. On the first and second of the four subjects the observations were terminated on the tenth and seventh days; consequently it is uncertain whether or not the total nitrogen had reached a steady state. But for the other two cases it certainly did not descend farther on the sixth and seventh days after starting the low N diet. Martin and Robison ('22) found that their own N-excretion fell in 5 days from 8.61 gm to 2.48, and from 8.79 gm to 1.99 daily for the two authors, respectively, when they started a "no-protein" diet.

In the present series of studies the "no-protein" period always changed the nitrogen intake abruptly from a level varying from 3.8 to 5.5% (of calories) for protein in the diet (see table 2), or from 4.5 to 6.7 gm N daily. The "no-protein" diet never contained more than 0.43 gm N, average for the squad, and was as low as 0.195 gm daily. Hence the absolute change was of the order of 4.3 to 6.3 gm N—a mean drop but little more than a third of that imposed by Folin. Expressed in percentage the average, however, was 94 compared with 93 in Folin's subjects. The starting level having been maintained usually for several previous 5-day periods and having often approximated much more nearly the endogenous level of excretion, it is fair to assume that much less reserve protein was present in these subjects than in Folin's and therefore that they would reach the endogenous level of excretion considerably earlier. This proved to be the case. In an early group of eight subjects, the average difference of urinary N between the second and third "no-protein" days was 0.378 gm and for half the squad who continued to the fourth day the difference between the third and fourth days was only 0.157 gm. In the next squad the third-day excretion proved to be slightly higher on the average than the second.



It was therefore decided to accept tentatively the excretion of the third day on "no-protein" as approximating closely enough the true endogenous level to insure dependable comparisons of the biological values of test proteins and corresponding mixtures of amino acids fed a little above this level. Later it was found, as expected, that the position of the no-protein period in a series of periods on comparatively low protein determined the reliability of the endogenous level of excretion attained by the third day. These facts will be illustrated below.

*(2) Position of the no-protein period in the series  
and nature of the protein preceding it*

A summary of the average endogenous nitrogens on the third day for 12 no-protein periods, and on the fourth day as well for four such periods is presented in table 2. The average daily fecal nitrogen excretions for the entire periods are included under the heading "endogenous" with full realization that the fecal nitrogen cannot be all "metabolic," to use Thomas' awkward term, but derives principally from residues of the digestive secretions and from exfoliated epithelial cells much of it transmuted into bacteria.<sup>2</sup>

In column 2 of table 2 is given in Roman numerals the number in each series, of the "no-protein" periods. It can readily be seen that later such periods in series 5, 6 and 8 (the only ones in which more than one occurs) gave the lower urinary nitrogens, and in 2 out of 3 of the series lower fecal nitrogens as well. This lower excretion rate, however, might well be influenced by the nature of the food protein which preceded the "no-protein" period in any given case, the level at which it was fed, and the length of time. Columns 3, 4 and

<sup>2</sup> For this reason this laboratory has preferred the term "alimentary nitrogen" which at least correctly indicates its origin if not its exact nature. It is a loss from the body (alimentary organs) incurred in the processes of digestion of the indispensable energy-giving foods and must be made good for maintenance. In that sense it represents maintenance as truly as do the products of catabolism of hematopoietic organs or the production of blood corpuscles themselves, the overturn of which must represent no small part of endogenous urinary nitrogen.

5 supply information with respect to these factors. Fortunately in series 6 and 8 the first two of them could be made identical; namely, whole egg protein and 5%-of-calorie level in all three periods. This circumstance leaves the factor of

TABLE 2

*Endogenous nitrogen excretion following different proteins and at different times in a series.*

SERIES NO.	NO-PROTEIN PERIOD	PROTEIN PRECEDING NO-PROTEIN	PROTEIN LEVEL AS % OF CAL.	NO OF DAYS OF THIS PROTEIN	AVERAGE ENDOGENOUS NITROGEN PER DAY		SUBJECTS No.	Body wt. (av.)	COEFFICIENT OF ENDOGENOUS N IN MG/KG OF BODY WEIGHT	
					Urine	Feces			Urine	Feces
					gm	gm		kg		
2	VI	Whole egg	4.0	4	3) 2.678	0.888	6	60.8	41.7	14.5
3	X	Amino acid mixture	3.8	5	3) 2.350	0.822	7	63.8	37.7	12.9
4	VI	Whole egg	4.0	6	3) 2.629	1.074	11	59.8	42.9	18.0
5	II	Whole egg	4.0	8	3) 2.642	1.201	10	66.6	39.6	18.0
	VII	Beefsteak	5.5	3	3) 2.403	0.959	10	66.0	36.8	14.5
6	II	Whole egg	5.0	11	3) 2.203	1.082	4	62.9	35.0	16.0
	VI	Whole egg	5.0	6	3) 2.131	1.227	4	63.0	33.8	19.5
	X	Whole egg	5.0	6	3) 2.169	1.288	3	64.6	33.6	20.2
8	II	Whole egg	5.0	9	3) 3.177	1.270	8	66.2	48.0	19.2
					4) 2.947			66.2	44.6	
	VI	Whole egg	5.0	7	3) 2.596	1.064	8	65.2	39.8	16.5
					4) 2.149			65.0	33.1	
	XII	Whole egg	5.0	7	3) 2.063	1.028	8	64.3	32.1	16.3
					4) 2.042			64.1	31.7	
9	VIII	Wheat germ	6.0	4	3) 2.379	1.026	6	68.1	34.9	15.1
					4) 2.050			68.1	30.1	
		Weighted av. 85 cases			3) 2.502			64.3	38.9	
		Weighted av. 22 cases			4) 2.083			65.5	31.8	

<sup>1</sup> 3) and 4) refer to days of no-protein period.

position unencumbered except for the length of time the preceding protein was fed. The latter may be resolved at once by pointing out that "no-protein" II necessarily is the one following the introductory protein after these squads had been for at least 2 months (series 6) on ad libitum diets. It was necessary therefore to prolong period I until the excretion

level no longer reflected the higher protein ingestion. A shorter period would certainly have produced a still higher "endogenous" excretion on any early day.

The issue with respect to the nature of the preceding protein (meaning its biological value) demands closer attention in the case of series 5, where preceding period II whole egg was fed for 8 days at a 4% level and preceding period VII beefsteak at 5.5% was fed for only 3 days. It appears that higher level could not compensate for lower value and a shorter period in sustaining the N excretion. In accordance with the usual interpretation this means that the protein reserves of the body had not been so well replenished by beefsteak as by egg, consequently were more quickly exhausted. Possibly a period of feeding equal to that of the egg period would have given a different result, to which the answer, not wholly adequate, is that beefsteak was itself preceded by egg for 2 days. Here we get into the question of more antecedent conditions than the diet immediately preceding, and for further light on this situation we must invoke a factor which is cumulative (table 3). The possible influence of an amino acid mixture (series 3), and of wheat germ, supplying a comparatively low value protein (series 9) likewise cannot be resolved without reference to antecedent conditions because each of these stands alone in the series; there is no endogenous period with egg as "supporting protein" preceding it with which either of these foods can be compared for the same squad of subjects.

Meantime it is noteworthy how remarkably well the "endogenous" urinary nitrogens agree following egg at 4% level, in two periods VI (series 2 and 4) and one period II (series 5). The average third-day excretions from two entirely different squads and a repetition with one, launched into no-protein from the same platform, so to speak, were 2.678, 2.629 and 2.642 gm N, respectively. How is it possible that egg protein could exert so constant an influence? The level of feeding was the same in the 3 widely separated periods, but the duration of this high quality influence varied from 4 to 8 days.

TABLE 3

*Relation of the sequence of proteins, and the consequent accrued nitrogen balance antecedent to no-protein, to the endogenous urinary nitrogen excretion.*

SERIES NO.	PLACE OF NO-PROTEIN PERIOD	SEQUENCE OF PROTEINS FROM BEGINNING OF SERIES TO NO-PROTEIN PERIODS, AND NO. OF DAYS OF EACH	FROM BEGINNING OF SERIES TO 3RD AND 4TH DAYS OF NO-PROTEIN				AVERAGE ENDOGENOUS URINARY NITROGEN FOR	
			Total days to		Accrued N. balances on			
			3rd day	4th day	3rd day	4th day	3rd day	4th day
2	VI	Egg 5, soy bean 4, egg 5, soy bean 4, egg 4	24		— 11.3		2.678	
3	X	Egg 6, yeast 4, egg 5, yeast 4, egg 5, yeast 6, beef 6, egg 6, amino acids 5	49		— 25.3		2.350	
4	VI	Egg 5, yeast 4, egg 5, yeast 4, egg 6	26		— 24.1		2.629	
5	II	Egg 8	10		— 11.9		2.642	
	VII	(No-prot. 3), cotton seed 5, amino acids 3, egg 2, beef 3	26		— 17.8		2.403	
6	II	Egg 11	13		— 3.3		2.203	
	VI	(No-prot. 3), corn germ 5, wheat germ + amino acids 4, egg 6	31		— 5.9		2.131	
	X	(No-prot. 3), egg 5, amino acids 4, egg 6	49		— 10.2		2.169	
8	II	Egg 9	11	12	— 12.9	— 16.6	3.177	2.947
	VI	(No-prot. 4), corn germ 5, amino acids 4, egg 7	31	32	— 36.7	— 40.4	2.596	2.149
	XII	(No-prot. 4), wheat germ 7, corn germ 9, amino acids 4, egg 7	62	63	— 59.9	— 62.8	2.063	2.042
9	VIII	Egg 6, egg 5, veg. mixt. + soy bean 4, wheat germ 4, veg. mixt. + soy bean and wheat germ 4, egg 5, wheat germ 4	34	35	— 13.3	— 16.5	2.379	2.050

Is this factor of duration then of no consequence? The answers will be found in the next section.

*(3) Cumulative antecedent conditions*

From a study of the data of Mitchell and associates in four of their publications from 1926 to 1933 Ashworth ('35) found considerable evidence that the previous diet influences endogenous nitrogen of the urine in rats. If high quality protein (liver) was fed immediately before the no-protein period the endogenous N in the urine rose above the previous level; if low quality protein (flour, cocoa) preceded the no-protein period the tendency was for that N to fall below previous levels. He found evidence of the influence of dietary protein on endogenous N excretion also in the work of Mason and Palmer ('35). In his own work, however, using lactalbumin as high quality and corn gluten as low quality protein Ashworth did not find any significant difference in the effect of these two on a final endogenous period, except "when the reserve protein supply of the body was reduced to a low level by long periods on N-free diets." He concluded that long periods on low quality test proteins at low levels of intake should not be used, if a decrease in endogenous N in a subsequent period is to be avoided.

Ashworth does not suggest, nor have we seen elsewhere any proposed means of measuring the effects of previously ingested proteins by which investigators, on whom is laid a responsibility for protection of their subjects, could predict the reliability of the endogenous level attained on a given day. The accrued nitrogen balance, as a criterion suitable for this purpose, seems to meet the need. For as a rule a balance can be struck for all previous food periods before the end of the third day of the current no-protein period and one can then determine whether an additional day is required. In these studies continuous nitrogen balances were available after the first few days of each series during which the subjects were becoming adjusted gradually to low (egg) protein.

Table 3 presents (col. 3) the sequence of proteins fed, in all of the series where endogenous nitrogen was especially needed, previous to its determination. In column 4 are found the elapsed times from the beginning of the series to the third and fourth days of the no-protein period in question, and in column 5 the accrued N-balances to those points. In each sequence the last protein named is the one given in table 2, column 3.

Referring to the questions raised in the previous section, we learn from table 3 that up to the third day of no-protein period VI in series 2 and the same day of no-protein period II in series 5, the total antecedent conditions had brought the two squads to the same average nitrogen deficit; namely, 11.3 and 11.9 gm, respectively. The supporting protein and its level being the same, the same endogenous excretion resulted.

The duration of the "supporting protein" period, as we have called the one immediately preceding a no-protein period, we thus learn cannot be the determining factor because the time it was fed in one case was twice that in the other. Neither can the nitrogen debt incurred previous to no-protein be credited with sole responsibility for the urinary N output; for while this item agrees perfectly for the two periods just mentioned, we find more than double the amount (24.1 gm) had been incurred up to the third day of period VI of series 4 in which the excretion of this squad of 11 averaged 2.642 gm — very close, as just observed, to the average performance of squads 2 and 5 on the corresponding day of their first no-protein periods.

It should be pointed out that squad 5, the subjects of series 5, are the same men, less one, who made up squad 4. They concluded the latter series with an accrued deficit of 23.1 gm of nitrogen each. Then intervened a recuperation period of 10 days in which it is safe to say they ate to repletion, so that the introductory period on egg in series 5 found their reserves well stocked. A careful analysis of all the data does not reveal any hold-over effect from the deficit incurred in series 4.

Almost certainly such a condition would have caused heavy retention and a positive balance in period I. Actually this period of 8 days on egg contained two short periods in which the balances were kept and both were negative.

The best conclusion possible at the moment is that egg protein is of such a high quality in building up reserves that, regardless of the length of feeding and the antecedent conditions affecting nitrogen equilibrium, it maintains as "supporting protein" a surprisingly constant third-day endogenous excretion and, as may be seen in figure 1, one that is higher

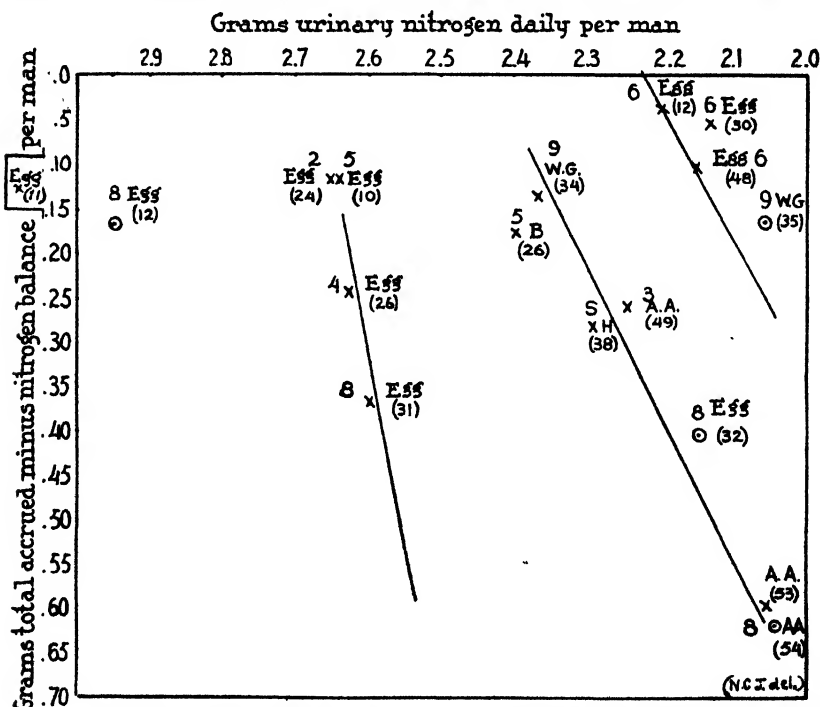


Fig. 1 Large numerals designate numbers of the squads whose daily averages are given by the coordinate points: x for third day of no-protein diet, ● for fourth day. A numeral in parentheses denotes number of days since beginning of the series to the day of no-protein designated. Name of protein is that given in table 2 as immediately preceding the no-protein period: W. G., wheat germ; B, beefsteak; H, halibut steak; A.A., a mixture of essential amino acids.

Halibut was fed to a special squad, designated "S", belonging to a different project.

than following any other protein we have used in that position. Four of the third-day determinations charted lie close together (to the left center) regardless of the magnitude of the accrued nitrogen deficit up to the day of measurement. The exceptional ones will be discussed in a moment.

The third-day endogenous values following other proteins used in the "supporting" position tend to group themselves to the right center of the chart and to be arranged also in a loosely linear fashion from upper left to lower right.

Series 6 deserves special consideration because it illustrates the fact that a squad of men, by subsistence over a very long period on low but fairly good protein on the average, may be brought to so low a state of reserve protein that starting as usual with egg as introductory protein and thus establishing at once a fair rate of nitrogen retention (aside from the "no-protein" periods) may still have reserves low enough to permit a steady endogenous level to be reached in 3 days. The squad started with 5 members, but one had to be excused at the end of 3 weeks because of a stomach ailment later diagnosed as gastritis. The remaining four had all been members of squads 4 and 5 (Aug. 15 to Dec. 22, 1944 with one break of 10 days) and two of them of squad 3 (June 10 to Dec. 22, 1944 with an additional break of 2 weeks). All had been off the diets from Dec. 22 to March 3.

The coefficients in milligrams of endogenous urinary N per kilo of body weight (table 2), prove that the average output for these men in period II of series 6 was already significantly lower than those of series 4, period IV, and series 5, periods II and VII, of which they had been members. Coincidentally their partial averages in the larger squads were exactly the same as those of the entire squads. It seems perfectly clear that their reserves had not been completely restored<sup>3</sup> when series 6 was begun.

<sup>3</sup> These men through January and February, 1945, had been subjects several times a week of hot-room experiments in another department, sweating profusely for many hours at a time, and had lost some weight.



Alarmed a little at this condition when it was discovered in period VI, blood serum proteins and hemoglobin values <sup>4</sup> were determined on them immediately after period VII with the result that all were found to be well within the normal ranges.

It is certain, therefore, that the liver and other hematopoietic tissues of these men had not suffered injury; but it is conceivable that the lower rate of endogenous excretion did not reflect a state of general depletion so much as an acquired power of conservation of such reserves as were available. The latter seems the more probable.

From March 3 until August 22 these four men continued on the low protein diets with a break of 17 days in May. Period II of series 6 found them with an accrued N-balance very near equilibrium, and their endogenous N for the third day at only a slightly higher average level than it was in period VI. In fact, as may be seen in figure 1, all three of the endogenous determinations lie very close together in the upper right-hand corner of the chart.

There is evidence in the column for fecal N in table 2 that these subjects did not digest protein so well as they had done in previous experiments; for their partial averages in this regard were also equal to those of the entire squads 4 and 5. This slightly higher excretion of fecal nitrogen, however, does not compensate for the lesser urinary excretion compared with earlier squads. It may indeed reflect only the higher intake that prevailed earlier in the supporting position.

Series 7, like series 1, contained no endogenous periods.

Series 8 started with a perfectly fresh squad of nine men, but the results are compiled for only eight of them, as one was lost by illness <sup>5</sup> before period XII. Since only about

<sup>4</sup> The authors are indebted to Mr. Robert Tully for these determinations.

<sup>5</sup> This man had arrived with furunculosis which persisted in spite of conserve drainage, but eventually appeared to be completely healed. Meantime, however, pain in the left ischial region developed and x-ray examination revealed "an area of bony erosions along the lateral margin of the left ischial tuberosity 1½ cm in depth," and a diagnosis of osteomyelitis was made. He was immediately admitted to the Orthopedic Division of Strong Memorial Hospital for penicillin treatment.

11 weeks remained before the then termination date of this project, we did not hesitate to subject these fresh subjects to three no-protein periods of 4 days each. The first of these found them with a slight minus balance which, augmented by the first days on no-protein, amounted to 12.9 and 16.6 gm N for the third and fourth days, respectively. Period VI began with an accrued deficit at 31 days of 36.7 and at 32 days of 40.4 gm. The endogenous urinary nitrogen on the fourth day (thirty-second from beginning of the series) approached a steady state as proved by period XII 22 days later (q.v.). The urinary nitrogens of the third and fourth days, after 53 days on comparatively low protein diets and at about 60 gm N-deficit, were equal.

Series 9 started with seven subjects, six of them CO men and one a parolee. Four were veterans from at least four earlier series, one a former subject returning after over 6 months respite, and two perfectly fresh dietary subjects. Because of the long tour of service on this project which the majority of them had given, only one no-protein period was imposed and that quite late in the series. At 32 days the accrued minus balance was only 3.79 gm and yet on the fourth day the average endogenous level of excretion was the same as for squad 8 after 51 days and an accrued minus balance of 46 gm. The difference is due to the better average state of nutrition of squad 8 at the start. The two squads were being studied simultaneously.

Summarizing this section it can be asserted that a quite definite relationship exists between the accrued N-deficit and the rate of decline in endogenous urinary nitrogen. In figure 1 this is shown by a shift to the right of all values the lower they lie in the scale of deficiency. They do not, however, fall into a single linear arrangement, but into three different slopes depending on the original state of nutrition and on the quality of the supporting protein. The fresher the subjects, following egg, the farther to the left in the chart; the more depleted at the beginning of the series, and also following egg, the farther to the right. The higher the position in the chart,

the more resistant is the reaction to length of time on low protein intakes; while the lower the position, the less resistant. The influence of biological value in the supporting position is seen with squad 5. Instead of moving downward in the chart in changing from egg as support to beefsteak, these ten men move to the right, i.e., the bottom drops out of the supporting structure, so to speak; reserves are not sufficient, or are more tenaciously held, and lower endogenous levels are attained. The effect of a fourth day as compared with the third is illustrated by squads 8 and 9. Following egg the movement from third to fourth (x to ©) for 8 is down the endogenous scale much more rapidly than down the deficiency scale. The same applies to 9 following wheat germ.

The chart, we believe, justifies our conservative plan with human subjects. It is not necessary to subject men at once to a long period of protein starvation, thereby possibly endangering the most valuable reserves — those which maintain blood proteins inclusive of specific protective agents. It can more safely be accomplished by a gradual approach. Keeping these men on a level of 5% of calorie intake with comparatively high value proteins for 25 to 35 days brought them to the region of relatively stable endogenous excretion with only moderate depletion, compared for example with the rate of depletion in Deuel's case (Deuel, Sandiford, Sandiford and Boothby, '28). A blood examination<sup>4</sup> made on squads 8 and 9 in the final periods of their respective series revealed only one man slightly below the normal range for hemoglobin and all who were examined (two from each squad did not appear) well within the normal for serum proteins.

#### REGRESSION OF ENDOGENOUS URINARY N ON BODY WEIGHT

Figure 2 displays the relationship of endogenous urinary N to net body weight. The statistical constants for these curves are given in table 4.

It is clear that the fourth day is less variable than the third for subjects who have been for at least 30 days on low protein diets of which at least two contained whole egg protein.

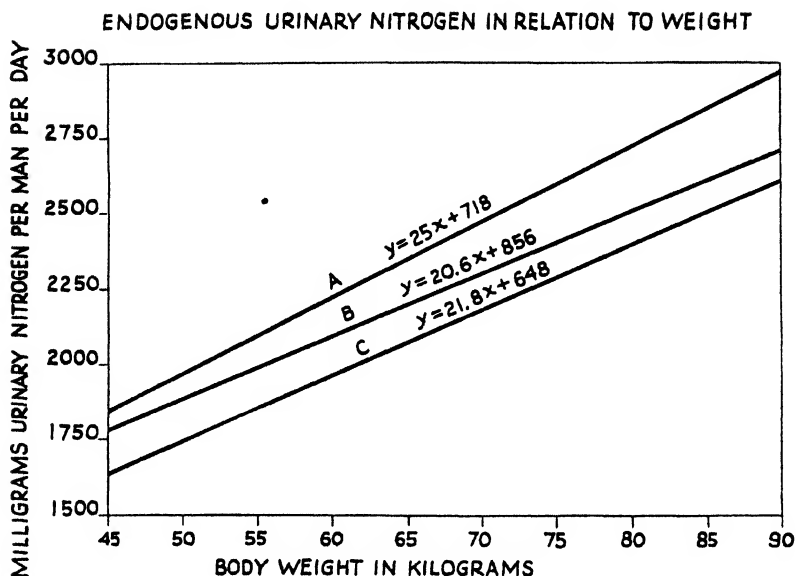


Fig.2 Regression curves described by their respective formulae for: A, 73 determinations of third-day no-protein excretion on 28 men; B, 7 determinations of third-day no-protein excretion on 7 women; C, 22 determinations of fourth-day excretion on 14 men.

TABLE 4

*Statistical data for plot of the relation of endogenous urinary nitrogen to body weight.*

Number	THIRD DAY		FOURTH DAY
	73 on men	7 on women	22 on men
Standard deviation	7.05	10.30	6.70 kg
Standard deviation	315.00	332.00	222.00 mg
Correlation coef.	0.56	0.64	0.66
Regression y on x	$25x + 718$	$20.6x + 856$	$21.8x + 647$

#### RELATION TO BASAL METABOLISM

It was discovered in 1927 by Terroine and Sorg-Matter that in several species of warm-blooded animals the total endogenous nitrogen excretion bears a constant relationship to basal metabolism. This was confirmed by Smuts ('35) for mice, rats, guinea pigs, rabbits and pigs, the ratios of milli-

grams urinary (only) nitrogen to calories of basal metabolism per 24 hours giving an average for the 5 species very close to 2.0. Brody, Procter and Ashworth ('34) have shown that the basal metabolism of the whole range of mammals from mouse to elephant increases with the 0.734 power of body weight while the endogenous urinary nitrogen for a series ranging from mice to cattle increases with the 0.72 power of body weight. Since the exponents of body weight on both log-log curves give slopes practically indistinguishable one from another, the authors conclude that "endogenous urinary nitrogen increases at the same rate with increasing body weight as does the basal metabolism." Exceptions to this nearly perfect harmony in the metabolic symphony, however, have been found in different stages of immaturity in rats by Ashworth and Cowgill ('38) and at different planes of nutrition by Treichler and Mitchell ('41).

It appears from the well-known experience of Deuel, Sandiford, Sandiford and Boothby that it requires a long time for a man to reach the minimal endogenous level of nitrogen excretion. After 39 days on a protein-free diet Deuel's urinary nitrogen was still falling slowly, while in the record of squad 8 (table 3) it required a period of only 29 days' continuous subsistence on a low protein regime, introduced by a 4-day period on no-protein, to reach the stage where, on the fourth day of the second no-protein period, the excretion appeared to become stable. Certainly 1 month later the excretion on the third and fourth days gave average levels for the entire group of eight men only 0.1 gm lower.

It seems incongruous that one phase of (protein) maintenance metabolism which cannot be attained inside a month should be so definitely ("rigorously" is the term used by Terroine and Sorg-Matter) proportional to another phase (energy) of maintenance metabolism which can be attained in 15 hours.

We have not been able to spare the time or to arrange for the use of the apparatus to follow basal metabolism in all our no-protein periods, as we should have liked to do; but

through the courtesy of Mr. J. T. Anderson and his assistant Mr. Frank Hastings we are able to present the suggestive data set out in table 5. Five of the eight men in squad 8 volunteered for basal determinations before breakfast on July 10, and again on August 10, just before closing the fourth day of the no-protein periods VI and XII in series 8 (table 3).

TABLE 5  
*Relation of endogenous urinary N to basal metabolism.*

SUBJECT	Wt.	Ht.	SUR- FACE AREA <sup>1</sup>	ENDO- GENOUS URINARY N IN MG/DAY	BASAL METAB- OLISM: CAL./DAY	ENDOGENOUS URINARY NITROGEN PER		
						Cal.	kg	sqm
	<i>kg</i>	<i>cm</i>	<i>sqm</i>					
July 10, 1945								
B.M.	51.8	167	1.58	1748	1315	1.33	33.2	1106
W.L.	57.2	170.7	1.67	1799	1411	1.27	31.4	1077
G.S.	65.3	167.5	1.75	2187	1217	1.80	33.5	1250
L.B.	71.9	167	1.80	2188	1488	1.47	30.4	1215
M.W.	72.0	179	1.90	2327	1507	1.54	32.3	1225
Av.	63.6	171	1.74	2049	1387	1.48	32.2	1173
							± 3.1%	± 5.6%
August 10, 1945.								
B.M.	51.8	168	1.58	1660	1294	1.28	32.0	1051
W.L.	56.4	170.7	1.66	1728	1410	1.22	30.6	1041
G.S.	63.3	167.5	1.73	1990	1306	1.52	31.4	1150
L.B.	70.0	165.5	1.77	2098	1498	1.40	29.9	1185
M.W.	70.4	180	1.89	2038	1555	1.31	29.0	1073
Av.	62.4	171	1.72	1903	1413	1.34	30.6	1101
							± 2.9%	± 5.0%

<sup>1</sup> By DuBois formula.

It is seen that the average weight had declined 1.2 kg from the first to the second observation, thereby affecting slightly the calculated surface area (DuBois chart). The basal metabolism by the Benedict-Roth clinical apparatus, with which Mr. Anderson has had extensive experience, was quite constant in the two determinations except for one subject (G.S.). The average endogenous excretion had fallen 146 mg — a little more than the average for the eight members of the

squad (table 3). The consequence is that the coefficient, endogenous nitrogen divided by basal metabolism computed to 24 hours, also fell from 1.48 to 1.34 mg per calorie.

But the important observation is that these coefficients are much below that found by Smuts, and agree well with the ratio 1.42 reached after 30 days on no protein in Deuel's experiment.

From the final columns it is seen also that the endogenous metabolism per unit of weight is less variable than when referred to a unit of surface by the DuBois chart.

We cannot escape the impression that the alleged proportionality between a stable endogenous urinary nitrogen and basal metabolism is somewhat less than "rigorously established for all homeotherms."

#### SUMMARY AND CONCLUSIONS

A basic diet is described containing (with arrowroot starch) not more than 0.2 gm N for the average individual, which has been used with but slight changes over a period of nearly 20 months. This diet constituted the "no-protein" regime in 12 different periods for determination of endogenous nitrogen excretion.

It was found that the following factors influence the time within which a dependable endogenous urinary nitrogen could be obtained within 3 or 4 days, on a no-protein diet: (a) the level of protein in the pre-experimental diets; (b) the position of the no-protein period in the series of periods; (c) the nature of the protein, called here "supporting protein," immediately preceding the no-protein, and its level of intake; and (d) conditions antecedent to the supporting protein which could affect the accrued nitrogen deficit to the beginning of the no-protein period.

Dependable and stable endogenous nitrogen levels can be reached on the fourth day of no-protein, even if the nature of the supporting protein and its level of intake provide a fairly stable deposit of reserve protein, but the total antecedent conditions bring the average subject, starting fresh, to a state of accrued nitrogen deficiency equal to about 40 gm.

Stable endogenous nitrogen excretion can be reached on the third day of no-protein provided the total antecedent conditions bring the average subject to a state of accrued deficiency equal to about 60 gm nitrogen. Some evidence has been developed that subjects acquire a resistance to the depleting effect of a no-protein diet after having passed through some months of low protein diets, including five or six no-protein periods.

Blood examinations for hemoglobin and serum protein prove that members of a diet squad passing through at least 6 months subsistence on alternating high and low value proteins at a general level of not to exceed 5% of the calories can withstand at least six no-protein periods of 3 days each within that period without apparent injury to the liver, so far as its protein-forming functions are concerned.

Regression equations are given of endogenous N on body weight in 73 determinations of the urinary excretion on 28 men and in one each on seven women for the third-day no-protein diet as well as in 22 determinations on 14 men for the fourth-day no-protein. The number of observations on women is too small to derive an entirely satisfactory equation.

In duplicate observations of basal metabolism on five men at the termination of the fourth-day no-protein in two periods 1 month apart, the average coefficient, milligrams endogenous urinary nitrogen to kg-cal. of the 24-hour basal heat, was found to be 1.48 and 1.34, respectively — values which agree much more closely with those of Deuel, Sandiford, Sandiford and Boothby and with two on women reported recently by Bricker, Mitchell and Kinsman ('45) than with those of Terroine or Smuts.

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# BIOLOGICAL VALUE OF PROTEINS IN RELATION TO THE ESSENTIAL AMINO ACIDS WHICH THEY CONTAIN <sup>1</sup>

## II. INTERCONVERTIBILITY OF BIOLOGICAL VALUES ILLUSTRATED BY SUPPLEMENTING EGG AND SOY PROTEIN WITH ESSENTIAL AMINO ACIDS

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### TWO FIGURES

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If two proteins of different composition are retained in different proportion because of this difference in composition, it might be expected that different effects would be obtained when the two proteins are supplemented by single amino acids added one at a time. The experimental demonstration consisted in giving to each member of the same diet squad of seven members a given amino acid in the same quantity, added first to the diet containing the egg protein at endogenous level (Murlin et al., '46), and in the next period to the diet containing the soy protein at (nearly) the same level. Table 1 shows how much of each of the seven acids was fed and the effect on biological value (B.V.) of the two proteins. Averages of the individual effects given in this table are summarized in figure 1. There was a definite increase in

<sup>1</sup> This study was carried out under contract with the Committee on Medical Research of the Office of Scientific Research and Development.

B.V. for only two of the seven added to egg at this level; namely, histidine and lysine, and for only one of the seven added to soy protein; namely, lysine. The latter gave the larger effect with soy, but the smaller effect with egg. It has been known since the work of Mitchell and Smuts ('32) and of Hayward, Steenbock and Bohstedt ('36) that soy protein, at least before cooking, lacks a sulfur-containing amino acid. Additional methionine to that already contained in baked defatted soy flour as in this case does not improve the B.V. The negative effects from threonine, valine and leucine, all of the racemic form, are strikingly different. Each test is the result of a 5-day experiment.

TABLE 1

*Effect of individual amino acids on biological value (B.V.) of egg and soy proteins.*

SUBJ. <sup>1</sup>	B.V. EGG ALONE	B.V. EGG + AM ACIDS	DIF. B.V.	B.V. SOY PROT. ALONE	B.V. SOY + AM ACIDS	DIF. B.V.	AMT. N ADDED GM	KIND OF AMINO ACID ADDED
2	85.9	85.4	- 0.5	86.1	75.6	- 10.5	0.5	<i>dl</i> Threonine
3	88.2	83.0	- 5.2	78.0	53.7	- 24.3	0.5	<i>dl</i> Valine
5	98.0	90.8	- 7.2	89.0	73.2	- 15.8	0.5	<i>dl</i> Leucine
7	90.4	91.3	+ 0.9	75.6	70.9	- 4.7	0.5	<i>dl</i> Methionine
8	101.0	93.4	- 7.6	85.3	66.2	- 19.1	0.25	<i>dl</i> Tryptophane
11	75.4	86.7	+ 11.3	75.0	55.6	- 19.4	0.25	<i>l</i> Histidine
12	95.5	99.6	+ 4.1	78.4	88.4	+ 10.0	0.25	<i>l</i> Lysine

<sup>1</sup> The original numbers for members of the squad are retained. Numbers 1, 4, 6 and 9 had to be excused for various valid reasons.

It is clear that adding equal quantities of the same amino acids to two different proteins eaten by the same squad gives quite different effects on B.V.; namely, an average reduction of less than 1.0 point for egg and 12 points for soy.

Figure 1 reveals some additional points of interest. The first group of columns gives the absolute biological value of egg protein. The first two show the difference between the nitrogen excretion on egg protein and on no-protein; the last two the difference for urinary nitrogen; and the middle two how the corrections for these differences are applied to food nitrogen eaten and food nitrogen absorbed, to get the percentage retained.

The next group of columns concerns the effect of superimposing a single amino acid onto the diet of each member of the squad getting egg at the endogenous level, which has just been discussed for individual effects. Now, we are concerned with the average effect, as a demonstration of reliability. Note that the average B.V. of the egg plus amino acid is 90.6% computed in the same way as the value of 92.1 is obtained, and from the same endogenous values. With these

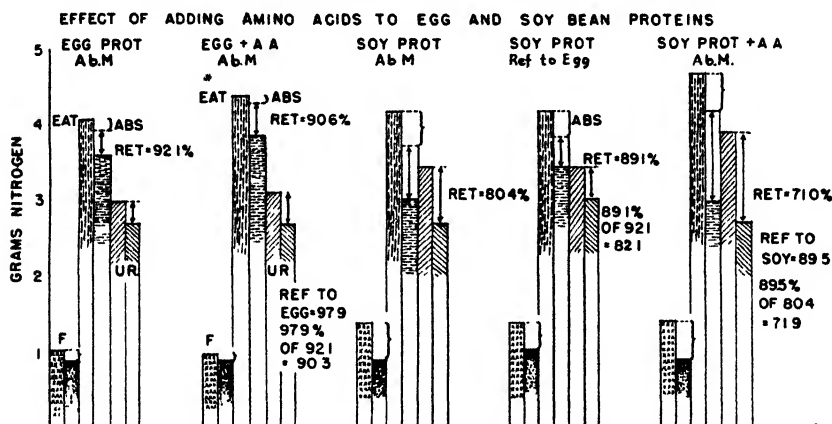


Fig. 1 These figures represent the quantities and operations necessary to obtain the biological value of a protein from two periods of feeding: (1) a no-protein diet and (2) the same diet plus the test protein. The two columns marked F represent the average excretion of a squad of seven members from the two diets in reverse order to the feeding. The last two, marked UR, represent the average excretions of urinary nitrogen from the two diets. The middle columns illustrate the two corrections to the food nitrogen. The small bracket, representing the excess of fecal nitrogen from the protein period over that from the no-protein period, corrects the protein nitrogen eaten (EAT), by the amount of waste ascribable to the food protein, to give the nitrogen absorbed (ABS.) from the protein. The two-pointed arrow, representing the excess of urinary nitrogen from the protein period over that from the no-protein period, corrects the absorbed nitrogen by the amount of waste ascribable to the absorbed food nitrogen, to give the retained (RET.) nitrogen, and this expressed as a percentage of the absorbed is the biological value. Ab. M. over a group of columns means "absolute method"; namely, estimating the B.V. of a protein against a true endogenous excretion (no-protein) level of both urinary and fecal nitrogens. Ref. to Egg means the same excretion data from a diet containing another protein, or a protein plus added amino acids, referred to the excretion data from a diet containing egg. In figure 1 the egg feeding referred to was at (or near) endogenous level; in 2 the egg feeding at  $2 \times$  endogenous level is used as base.

data available we now calculate the B.V. of the combination egg plus amino acid in terms of the excretion data on the egg protein alone. In other words, we are now using egg protein instead of body protein as the basis. The result tells us that this combination is 97.9% as good as egg protein alone.

Egg plus amino acid being 97.9% as good as egg for retention of nitrogen to replace body waste, and egg protein being 92.1% as good as the hypothetical perfect protein the combination should (in the absolute scale) be worth 97.9% of 92.1 which is 90.3. This compares very well indeed with 90.6 for the absolute B.V. determined directly. Obviously biological values based on egg protein are convertible to the absolute scale and therefore become just as reliable as the absolute value directly determinable. One needs no statistical aid to arrive at a feeling of satisfaction with this outcome. The agreement could not be so good if the fundamental data were not consistent and reliable.

Applying the same methods and reasoning to the parallel study of soy protein, we arrive at: (1) 80.4 for the absolute biological value directly determined; (2) 89.1, referred to egg as standard; and (3) 82.1 instead of 80.4 for the absolute value, indirectly determined (i.e.,  $89.1\% \text{ of } 92.1 = 82.06$ ). This result is not quite so satisfactory.<sup>2</sup>

The final comparison on the chart is one involving the direct absolute B.V. for soy plus amino acids, and the value determined indirectly. Direct value is 71.0 — which of course means that adding single amino acids to a medium-value protein does not effect any improvement; rather the contrary. Referred to soy protein alone as a base, we find that the soy plus amino acid is only 89.5% as good. Soy on the absolute scale being 80.4 and soy plus the additions measuring only to 89.5% of soy, the average absolute B.V. of the combinations,

<sup>2</sup> If the absorption of soy protein in relation to egg had been the same as its absorption in relation to endogenous excretion (in other words, if the correction on fecal nitrogen had been the same), the converted B.V. would have been 81 instead of 82.1.

indirectly determined, is (89.5% of 80.4 =) 71.9.<sup>3</sup> In summary we have: for egg plus amino acids 90.6 direct, 90.3 indirect; for soy protein 80.4 direct, 82.1 indirect; and for soy plus amino acids 71.0 direct, 71.9 indirect.

*Effect of level of intake of the supplemented protein*

The results just described were obtained with the members of a diet squad after they had been considerably depleted of nitrogen not only by the no-protein period, but by the subsequent periods on soy protein. The supplementing effects of single essential amino acids in the amounts fed (as shown in table 1) were largely negative so far as the biological value referred to the endogenous nitrogen was concerned, and they were quite different when added to egg protein and to soy protein at the endogenous level in the same amounts. The negative effect could scarcely be due to depletion alone for this would tend to enhance retention. We must suppose that when one of the essentials is added under these conditions and produces a negative effect whatever the protein supplemented, it is not needed under the circumstances — which may be another way of saying that it is not qualitatively adapted to meet the needs of the moment.

For the purpose of testing the opposite condition, namely, repletion of protein and consequent high plus nitrogen balances, on the effects of supplementation with amino acids, the squad was next placed on a level of egg protein intake approximately double that prevailing in the above four periods, namely, 7.79 gm N or 46.8 gm protein daily (including 0.3 gm N in the remainder of the diet) instead of 4.073 gm N. The average retention for the last 3 days of this diet was 1.76 gm N daily. This reflects the influence of the soy period immediately preceding when the average balance was

<sup>3</sup> If the average absorption rate of soy protein plus amino acids had been the same in relation to soy protein alone, as it was in relation to the endogenous excretion, this value would have been 70.9. Actually there was a little tendency to diarrhea on this period with amino acids and the fecal nitrogen which usually is reduced when amino acids are added to a protein was increased a little. This phenomenon of reduction will be considered in a later paper of this series.

—0.611 gm N daily. Retention of nitrogen from egg far more than made up the deficit.

When the supplementary amino acids were again added (in the next period) in equal amounts as before, there was a marked decrease in nitrogen balance in all subjects in the last 3 days of the period. Some of this decrease probably would have occurred without the amino acids, because the retention demand had been satisfied. But it is of special interest: (1) that all of the seven amino acids fed produced effects in the same direction; and (2) that this reduced retention in every case but one (leucine) exceeded the nitrogen

TABLE 2

*Change in nitrogen balance when egg protein at two levels of intake is supplemented with amino acids.*

(Same subjects received same amino acids in same amounts.)

GM N FROM	AT ENDOGENOUS LEVEL			AT DOUBLE ENDOGENOUS LEVEL		
	Egg alone	Egg plus am. acid	Dif.	Egg alone	Egg plus am. acid	Dif.
	gm	gm	gm	gm	gm	gm
0.5 dl Threonine	— 0.042	+ 0.139	+ 0.181	+ 1.846	+ 1.124	— 0.722
0.5 dl Valine	+ 0.122	+ 0.389	+ 0.267	+ 1.401	+ 0.866	— 0.535
0.5 dl Leucine	+ 0.350	+ 0.212	— 0.128	+ 1.504	+ 1.047	— 0.457
0.5 dl Methionine	— 0.217	+ 0.034	+ 0.251	+ 2.190	+ 1.409	— 0.781
0.25 dl Tryptophane	+ 0.652	+ 0.661	+ 0.009	+ 2.583	+ 1.762	— 0.821
0.25 l Histidine	— 1.483	+ 0.520	+ 2.003	+ 1.447	+ 1.016	— 0.431
0.25 l Lysine	— 0.004	+ 0.192	+ 0.196	+ 2.152	+ 1.795	— 0.357

contributed by the amino acid. It appears, therefore, that with the exception of a slight effect from leucine none of the amino acid nitrogen added as a supplement under these circumstances is so available for tissue repair as nitrogen from the egg protein.

Table 2 brings together for comparison the effects on nitrogen balance from supplementing with the same amounts of the seven essentials fed to the same persons, several periods apart: (1) at the endogenous level and (2) at approximately double this level. At the lower level *dl* leucine is the only one that produced a negative effect on daily nitrogen balance as

compared with the period on egg unsupplemented. At the higher level all produced a negative effect. As already noted these reduced retentions were greater than the amounts of nitrogen contributed by the several supplements, whereas in every case of supplementation at the lower level the extra

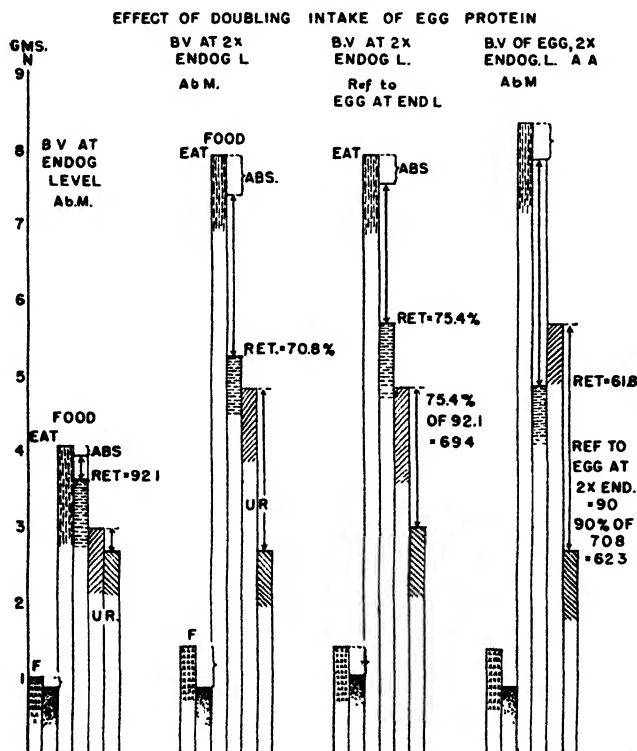


Fig. 2 The statement under figure 1 also applies to figure 2.

retention (when it occurred) was less than the amount of nitrogen contributed.

The effects of supplementation on the B.V. will be evident from the last columns of figure 2. The first group of columns on the left is the one seen in figure 1, picturing the average data obtained in a 6-day experiment on the seven subjects, for the endogenous B.V. of egg protein at an average level of



4.073 gm N. The second group gives the results on the same squad with the same basal diet, but with the egg protein at double the endogenous level. The fecal nitrogen on this higher intake is considerably elevated above the other. Comparing with the same endogenous fecal nitrogen, a larger waste is evident. However, deducting this from nitrogen ingested, the value for absorption is 96% against 97% for the endogenous level—digestibility, therefore, is not reduced materially. Urinary nitrogen shows a much larger wastage both in absolute and relative terms. The retention therefore is only 70.8% of absorbed, against 92.1. This is the B.V. of the higher intake of egg protein: 71 in round numbers compared with 92 for the lower level.<sup>4</sup>

Referred to the lower egg intake as standard (third group of columns) the B.V. of the higher feeding comes out 75.4, and 75.4% of 92.1 is 69.4—not a bad agreement with 70.8, directly determined.

Adding the seven individual amino acids in the same amounts with respect to the endogenous level and thereby raising the nitrogen intake by only 0.39 gm we find an absolute B.V. of 61.8. Referring now to the egg intake at double endogenous as standard the B.V. is 90, and 90% of 70.8, the absolute value of double egg, the derived absolute, is 62.3. Thus the direct value is 61.8, the indirect 62.3. The agreement could hardly be better. The interconvertibility of biological values from endogenous base to the metabolism data from egg protein at that level of intake, and even to similar data obtained at double this intake, is clearly demonstrated.

*Conversion of a relative B.V. of egg at one level against egg at another (endogenous) level by means of a conversion factor obtained on another squad*

All the conversions from one base to another thus far considered were calculated from data obtained on the same squad

<sup>4</sup> As regards the effect of different levels of feeding the same protein on biological value, there is of course nothing new about these results. Hamilton ('39) has shown that feeding egg protein to rats at a level of 4% (of dry weight) gave a B.V. of 100; at 8% it was 91; at 12% 84; at 16% 62; and so on.

of subjects, but as shown in table 3 it can be just as readily accomplished from the general data obtained on one squad by means of a conversion factor obtained on another. This is not mere coincidence, for the same type of conversion has been obtained between other squads, as will be described in another communication.

The absolute B.V. obtained on squad 4 was referred to the endogenous nitrogens from that squad. It is lower than the value would have been if egg had been fed at a level nearer

TABLE 3

*Conversion of a relative biological value (B.V.) of egg at one level of intake against egg at another (endogenous) level by means of a conversion factor obtained from experiments with another squad of human subjects.*

		EGG AT 5% OF CALORIES	
		B.Vs. endogenous excretion	B.Vs. egg at endogenous level
(a)	Test protein fecal N	1.269	1.269
(b)	No-protein fecal N	1.074	1.017
(c)	Fecal waste N (a-b)	0.195	0.242
(d)	Test protein N eaten	5.798	5.798
(e)	Absorbed N (d-c)	5.603	5.556
(f)	True digestibility ( $\frac{e \times 100}{d}$ )	95	95
(g)	Test protein urine N	3.264	3.264
(h)	No-protein urine N	2.629	2.989
(i)	Urinary waste N (g-h)	0.635	0.275
(j)	Retained N (e-i)	4.968	5.281
(k)	B.V. ( $\frac{j \times 100}{e}$ )	88.6 Abs.	95.0 Rel. $\times 0.921$ 87.5 Abs.

that of the net endogenous losses (urinary and fecal nitrogens less the nitrogen in no-protein foods). When the same 5% level was evaluated against the excretion data from egg at endogenous level of feeding, the relative value was converted to absolute by the factor 0.921 obtained from squad 2. This merely illustrates the fact that the data from one squad, differing entirely in personnel and nutritional history from the other, may, if reliable, be used to interpret data from the second. Successful conversion in fact becomes a measure of reliability.

## SUMMARY

1. Adding single essential amino acids to egg or soy bean protein (baked) at or near the endogenous level of intake does not improve the biological value of these proteins, rather the contrary—even though they may improve nitrogen balances.

2. Biological values of egg and soy bean proteins determined at or near endogenous levels (absolute method) may themselves be used as conversion factors to translate biological values of these proteins plus amino acids (referred to the proteins unsupplemented) to the absolute scale with very little error.

3. Likewise the absolute biological value of egg fed at double the endogenous level of intake may be used as a conversion factor to translate values relative to egg at this level to the absolute scale.

4. The conversion factor reliably determined on one squad of subjects may be applied also to the fundamental data of relative values obtained on another squad to convert the latter to the absolute scale.

5. Once these conversion factors are reliably determined they may be applied widely to relative values obtained in a proper sequence of feeding periods to bring the latter into line with absolute values, as will be shown in a later paper. Thus the existing confusion of values obtained at widely different levels of feeding can in time be completely resolved.

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# THE INFLUENCE OF PHOSPHORUS, CALCIUM AND VITAMIN D<sub>3</sub> UPON THE FAT CONTENT OF THE SKELETON IN GROWING PIGS

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The mechanism of bone calcification has been the subject of much discussion. Most studied in this connection are the elements Ca and P, the enzyme phosphatase, and the effect vitamin D exerts upon these elements in connection with the calcification process. As to the influence of vitamin D, it appears that the earlier view that this factor acts through increased intestinal absorption of phosphorus has now been modified. New experiments with radioactive phosphorus have given results which support the theory that vitamin D exerts its effect by intensification of phosphorus turnover in bone, accompanied by hyperphosphatemia and decreased visceral phosphorus turnover.

The effect of dietary fat upon bone calcification has been the object of several studies. In his work on the so-called "anticalcifying" action of cereals, Mellanby ('22 a; '22 b; '24; '26; Green and Mellanby, '28) suggested that this effect might be related to the fatty acids in the diet. Bunkfeldt and Steenbock ('43) studied the effect of dietary fat on bone calcification in rats receiving diets completely deficient in vitamin D. They observed that when the diet had a very low content of phosphorus the calcification of the femur was diminished by fat. When phosphorus was fed at, or above, optimum levels, dietary fat increased the calcification. When the value of the Ca : P ratio was above 4 the effect was not

observed. Other workers (Jones, '43) have also reported that fat in the diet improves the absorption of calcium. Therefore, it is quite clear that the presence of fat in the diet in any case does influence the mineral absorption. Regarding the influence of the absorbed fat upon the mechanism of ossification, this has received less consideration. As to the fat in the bone, most workers are of the opinion that this merely serves as a depôt for fat storage, especially the yellow fat in hollow or long bones. The red marrow within the ends of long bones and in the interior of flat ones is known to be the place where red blood-cell formation takes place (Carlson and Johnson, '37). It is also known, that in the severely anemic patient the active bone marrow is always increased in amount and may extend throughout the long bones (Minot and Strauss, '45). Hirsch and Weinhouse ('43) have reported a study of atherosclerosis, and this process showed a great similarity to normal bone formation. In their study Hirsch and Weinhouse summarized that the simple fatty deposits in atherosclerosis had the same lipid composition as blood plasma and the normal intima, and that after the lipids are deposited and remain in the tissue, the subsequent stages of atherosclerosis follow.

Against this incomplete picture of the lipid rôle in ossification, it is of interest to study how the lipid content of bone is influenced by certain factors in the food, such as mineral nutrients and vitamin D. In the experiments reported in this paper the influence of minerals and of vitamin D upon the fat content of the bone has been studied.

#### INFLUENCE OF PHOSPHORUS AND CALCIUM UPON THE FAT CONTENT IN THE BONES

The experiments were planned for the study of the influence of the diet upon the calcification process in growing pigs; several series of experiments were carried out. All the pigs used were of the Swedish Landrace breed. The diet in the experiment consisted of 50% ground corn and 50% ground oats and an average of 2.5 kg of skimmed milk per animal

daily. In order to satisfy the mineral requirement, from 20 to 40 gm of limestone and 5 to 10 gm of common salt (NaCl) were added daily. Vitamin A was provided through the carotenoids in the yellow corn; extra vitamin D was not given. The average percentage composition of this diet figured on the basis of dry matter, was the following: Crude fiber 6.5, nitrogen-free extract 68.6, crude protein 12.0, crude fat 3.8, calcium (Ca) 0.82, and phosphorus (P) 0.42. The analyses of the feed for these components were carried out by standard methods, in agreement with the regulation given by the Swedish Royal Board of Agriculture ('40).

The first experiment included 16 pigs divided as equally as possible into two groups, 1 and 2. The animals were divided according to litter, sex, weight and pre-experimental development. The experimental feeding began when the animals weighed 34 kg and finished at a weight of 100 kg, when the animals were slaughtered. During the period of the experiment, which covered more than 3 months, group 1 received the diet and the mineral supplements mentioned. The Ca : P ratio was 1.95. Group 2 received in addition 40-45 gm of di-sodium phosphate per pig daily. The supply of Ca and P for this group averaged 0.81% of Ca, and 0.58% of P calculated on the dry matter with a Ca : P ratio of 1.40. The development of the pigs during the experiment was controlled by weighing the animals at weekly intervals and by controlling the feed consumption. The daily average weight-increase in group one was 658 gm and in group two 719 gm. After slaughter one femur was taken out, prepared and analyzed for dry matter, crude fat and ash. The fat was determined according to the method of Schmid-Bondzynski-Ratzlaff ('03). The bones were prepared before the analysis in such a way that they were freed as far as possible from soft tissue such as fat, muscles and connective tissue by scraping the surfaces with a knife. After this the large bone was first crushed in a large meat-grinder (the Huskvarna "fox mill" no. 8), whereupon the material was homogenized in a special laboratory grinder. This method of preparation explains the rela-

tively high values of fat content in the bone. The method was used because of the large amount of material prepared. As is seen from the data given, this method gave, however, satisfactory results. The results of the analyses are summarized in table 1.

From the results given in table 1 it may be seen that under the conditions described extra phosphorus in the diet decreases the amount of fat in the bones. This effect, however, is not specific for phosphorus alone.

TABLE 1

*Fat content of the femur as affected by supplementing the diet with phosphorus and by vitamin D<sub>3</sub>.*

EXPERI- MENT NUMBER AND GROUP	NUMBER OF ANIMALS	REGIMEN	FEED				FEMUR <sup>1</sup>	
			(per kg dry matter) in grain and milk		supple- ments		crude fat (dry basis)	ash (dry basis)
			Ca	P	Ca	P		
			gm	gm	gm	gm		
VII		With and without	supplements of phosphorus <sup>2</sup>					
1	10	Without extra P	2.5	4.2	5.7	..	36.9 ± 0.85	62.7 ± 0.71
2	10	With extra P	2.5	4.2	5.6	1.6	32.1 ± 1.75	60.3 ± 1.46
IV		With and without supply of vitamin D <sub>3</sub> <sup>3</sup>						
1 a	4	With D <sub>3</sub>	1.8	4.0	..	..	41.9 ± 0.92	59.5 ± 0.53
1 b	4	Without D <sub>3</sub>	1.8	4.0	..	..	37.5 ± 1.01	59.5 ± 0.24
2 a	4	With D <sub>3</sub>	1.8	4.0	2.4	..	40.2 ± 0.72	61.2 ± 0.29
2 b	4	Without D <sub>3</sub>	1.8	4.0	2.4	..	34.0 ± 2.77	61.2 ± 0.51
3 a	4	With D <sub>3</sub>	1.8	4.0	5.4	..	38.5 ± 1.37	60.8 ± 0.95
3 b	4	Without D <sub>3</sub>	1.8	4.0	5.4	..	37.2 ± 1.32	60.7 ± 0.61
4 a	4	With D <sub>3</sub>	1.8	4.0	7.1	..	38.4 ± 1.01	61.8 ± 1.07
4 b	4	Without D <sub>3</sub>	1.8	4.0	7.1	..	36.5 ± 1.31	61.9 ± 1.05

<sup>1</sup> Values are given with their standard errors calculated from  $\Sigma = \frac{\sigma}{\sqrt{n}}$  where  $\sigma = \sqrt{\frac{\sum d^2}{n-1}}$ , "d" is the deviation from the average, and "n" the number of observations.

<sup>2</sup> The basal diet consisted of 50% ground corn, 50% ground oats and an average of 2.5 kg skimmed milk per animal daily; 20 to 40 gm limestone, and 5 to 10 gm common salt daily. Ca/P value was 1.95. Group 1 received the basal diet only; group 2 received in addition 40-45 gm Na<sub>2</sub>HPO<sub>4</sub> per pig daily.

<sup>3</sup> The basal diet consisted of 75% oatmeal, 25% corn meal, and 2.5 kg skimmed milk per animal daily. Group 1 received no extra minerals except NaCl; other groups received Ca in form of limestone. Ca/P values were: for group 1, 0.45; 2, 1.05; 3, 1.80; 4, 2.23. D<sub>3</sub> was provided in form of a concentrate called "Delta" which contained 200 I.U. per gm.

From feeding experiments made in Denmark in which minerals were fed to pigs Petersen ('43) has reported data for Ca similar to those given above for P; however, this effect of Ca or P is not mentioned in Petersen's paper. In his report Petersen has given the content of crude fat in the scapulae of pigs, fed on a diet of grain and skimmed milk with and without any addition of limestone. The diet contained 2.3 gm Ca and 4.8 gm P per kg of dry matter of the feed. In one experiment this diet gave a fat content of 14.4%, figured on the basis of dry matter in the bone. When 1.7 gm Ca was given as a supplement per animal daily, the fat content was lowered to 12.5%. In another similar experiment the fat content of the bone was lowered from 17.1% to 12.2%, when 3 gm Ca was supplied in the diet. In another case the supplement was raised to 9 gm Ca, and this reduced the fat content to 9.2 gm. The data cited represent the average values for 7 to 10 experimental animals. It should be noted that the bone investigated in this case was a flat bone containing red marrow. Evidently the observation holds true for red as well as for yellow bone-marrow fat.

Since it has been shown that phosphorus alone or calcium alone exerts the influence mentioned upon the content of bone-marrow fat, it might be expected that these two elements, when given together as supplements in the feed should likewise reduce the amount of crude fat of the bone. That such is the case is also indicated by Petersen's data. When no extra minerals were supplied in the diet mentioned, the fat content of the scapulae was 14.0% figured on basis of dry matter. When 6.4% Ca and 2.3 gm P were given daily as supplements to the feed, then the fat content in the bone in one experiment was only 10.3%. In another experiment 16.1% fat in the bone was obtained when no minerals were supplied. When 5.5 gm Ca and 1.0 gm P were supplied as minerals, 11.3% fat was obtained as average for the bone. Each value is based upon the results from 6 to 8 experimental animals. The Ca and P were added in the form of commercial dicalcium phosphate or bone meal.



INFLUENCE OF VITAMIN D UPON THE  
FAT CONTENT IN THE BONES

A series of experiments was carried out where the effect of vitamin D upon the fat content in the bone could be observed. The feed used during the experimental period consisted of 75% of oatmeal, 25% of corn meal, and 2.5 kg of skimmed milk per animal daily. Thirty-two pigs were used in the experiment; the animals were divided into four groups, each group containing 8 animals. The experiment began when the animals weighed about 45 kg and was continued until they weighed 125 kg, a period of about 4 months. Group 1 received no extra minerals except common salt; Ca in percent of dry matter in the feed averaged 0.18 and P 0.40. The other groups received extra Ca in the form of limestone, which increased the Ca-content of the feed as shown in table 1. The values for the Ca : P ratio were: group 1, 0.45; 2, 1.05; 3, 1.80; and group 4, 2.23.

Each group was divided into two subgroups including 4 pigs, where one subgroup received no supplement of vitamin D, and the other received a supplement of 1 gm of a preparation of D<sub>3</sub> containing 200 I.U. per gm. Consequently, 16 animals in the experiment received extra vitamin D, and 16 animals obtained no such supplement. In addition to other observations made during and after the experiment, the femur was analyzed for dry matter, ash and fat. The results of these analyses are given in table 1.

The results indicate that vitamin D<sub>3</sub> in its influence upon the fat content in bone has an effect opposite to that demonstrated for Ca and P. Thus, the fat content in the bones of all the groups reported in table 1 is higher when vitamin D is added to the diet than when no such vitamin is added. It is of interest to observe, however, that the difference in fat content between groups fed with and without vitamin D is much more pronounced where only small amounts or no supplements at all of Ca are given than it is when relatively large amounts of Ca are given. In groups 3 and 4, where 5 and 7 gm of Ca are given as supplements per kg of dry matter, the

difference in fat content in the bone is only 1.3 and 1.9%, while the corresponding figures in groups 1 and 2, which received only 2.4 gm or no Ca at all as supplements, are 4.4 and 6.2%. It appears therefore that, as far as the fat content of the bone is concerned, the influence of vitamin D has been counteracted by the influence of the extra Ca given to groups 3 and 4, so that in this case the special effect of vitamin D<sub>3</sub> is relatively small.

#### SUMMARY

1. Feeding experiments with pigs were carried out where the diet consisted of grain, such as barley, oats, and corn, and, in addition, 2.5 kg of skimmed milk daily per animal was given. In some cases Ca was added to the diet, in other cases P, and in other instances vitamin D<sub>3</sub>. The experimental period included a time of 3 months or more, and continued from a weight of the animals of 35–45 kg up to 100 kg or more. Among other observations which were carried out in connection with the experiment, the fat content of a bone of the skeleton of the animals was studied.

2. Calcium, or phosphorus, or both elements together, decreased the content of fat in the femur when the elements were added as supplements to the diet mentioned. This observation of ours made on a long, hollow bone, such as the femur, agrees with that made by Petersen on a flat bone, the scapulae.

3. Vitamin D<sub>3</sub> appeared to increase the amount of fat in the femur when this vitamin was given to the pigs under the conditions described. When other Ca or P was given simultaneously as a supplement to the diet the increase in fat content in the bone was less pronounced. It therefore appears as if Ca and P on the one hand and vitamin D<sub>3</sub> on the other counteract each other in this influence upon the fat content of the skeleton.

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# THE ASCORBIC ACID CONTENT OF WHOLE BLOOD PLASMA OF NORMAL RATS WITH EVIDENCE OF A SEX DIFFERENCE<sup>1</sup>

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An answer to the question of what is a "normal" level of ascorbic acid in human blood would aid in the determination of nutritional status. Although plasma values of zero have been reported without clinical signs of scurvy, there must be some blood level of ascorbic acid at which the body functions best, and values below that level might indicate malnutrition with regard to ascorbic acid.

A number of investigators have reported that values below 0.7 mg ascorbic acid per 100 ml of plasma indicate inadequate dietary intakes of ascorbic acid. Munsell et al. ('44) have raised the question of what, if any, level of plasma may be taken as indicative of vitamin C deficiency since determinations made in that laboratory on healthy animals receiving an adequate diet showed plasma values of 0.25 mg to 0.56 mg. Some plasma values reported for a number of different animals by various investigators are shown in table 1. These values are from apparently normal healthy animals receiving adequate diets. With the exception of the low values for the dog and high values for hens those for the other animals lie between 0.30 mg and 0.66 mg.

Rats synthesize ascorbic acid; therefore it might be assumed that plasma values of healthy rats maintained on an adequate diet would be an indication of normal values, at

<sup>1</sup>This investigation was made possible by grants from the General Education Board and the University of Alabama Research Fund.

least for that species. This investigation was undertaken to determine the level of ascorbic acid in the plasma of adult rats and since several investigators have suggested that the values for whole blood are more reliable than plasma values, deter-

TABLE 1

*Plasma ascorbic acid values for different animals as reported in the literature.*

ANIMAL	PLASMA ASCORBIC ACID	REFERENCE
	<i>mg/100 ml</i>	
Monkey	0.41	Munsell et al. ('44)
Guinea pig	0.56	Munsell et al. ('44)
Guinea pig (10) <sup>1</sup>	0.54	Todhunter and Brewer ('40)
Guinea pig	0.30	Karel and Chapman ('44)
Rabbit	0.41	Munsell et al. ('44)
Goat	0.46	Munsell et al. ('44)
Horse	0.46	Munsell et al. ('44)
Mare	0.53 ± .17	Rasmussen et al. ('44 a)
Sheep	0.56	Rasmussen et al. ('44 b)
Lambs	0.48 ± .21	Rasmussen et al. ('44 b)
Ewes (20)	0.66 (0.43-0.82)	Satterfield et al. ('42)
Dairy calves (19)	0.32 (0.03-0.77)	Bortree et al. ('42)
Dairy heifers (4)	0.49 (0.24-0.80)	Bortree et al. ('42)
Dairy cows (24)	0.44 (0.11-0.80)	Bortree et al. ('42)
Hens, 12 weeks age	2.05 (1.46-2.43)	Satterfield et al. ('45)
Dog	0.25	Munsell et al. ('44)
Dog, 2 mo.-6 yrs. (37 ♂)	0.353 (0.172-0.840)	LaCroix et al. ('42)
Dog, 2 mo.-6 yrs. (19 ♀)	0.368 (0.126-0.743)	LaCroix et al. ('42)
Dog, 6-14 yrs. (24 ♂)	0.353 (0.168-0.591)	LaCroix et al. ('42)
Dog, 6-14 yrs. (17 ♀)	0.308 (0.118-0.620)	LaCroix et al. ('42)

<sup>1</sup> Number of animals shown in brackets.

minations on whole blood were also made. The hemoglobin content was measured at the same time to see whether there was any relation between hemoglobin and ascorbic acid content of the blood.

## EXPERIMENTAL

### *Animals*

Albino rats from the breeding colony, raised on a dog chow,<sup>2</sup> supplemented once a week with lean meat or liver, and

<sup>2</sup> Purina Dog Chow Checkers.

lettuce or carrot, were used. The first group of animals were mature breeders; later, several litters were raised specifically for this study and were sacrificed at 250 gm weight for males and 200 gm for females.

Animals were anesthetized by intraperitoneal injection of a solution of 20 mg nembutal (Kuether, Telford and Roe, '44) and within 15 to 20 minutes an incision was made and the heart exposed. Approximately 5 ml of blood were withdrawn in a syringe and used for hemoglobin and ascorbic acid determinations.

### *Hemoglobin*

Hemoglobin determinations were made with a photometer<sup>3</sup> according to the method of Sheard and Sanford ('29). For each determination two aliquots of 0.02 ml of blood were used. The accuracy of the method was verified by comparison with results obtained by the oxygen capacity method of Van Slyke<sup>4</sup> (Van Slyke and Neill, '24).

### *Ascorbic acid*

Plasma ascorbic acid was determined by a modification of the micromethod of Mindlin and Butler ('38), omitting the use of KCN. In order to obtain the necessary volume for reading in the Coleman spectrophotometer 0.5 ml plasma filtrate and 1 ml buffered dye were used. Sodium-acetate buffer was used in such concentration (2.25%) to give a pH of 4.1 for the dye-plasma filtrate mixture; pH measurements were made with a Beckman pH meter. Corrections were made for turbidity. The addition of pure ascorbic acid to plasma analyzed by this procedure gave recoveries of 96.6 to 110.3%, which compare favorably with those reported in the literature (Mindlin and Butler, '38).

The method of Roe and Kuether ('43) was used to determine the ascorbic acid content of the whole blood, using 2 ml

<sup>3</sup> Cenco-Sheard-Sanford, Type C-5.

<sup>4</sup> We are indebted to Dr. J. M. Bruhn of the University of Alabama Medical College for making this determination.

of blood for each determination. Recoveries of added ascorbic acid by this method were 89.3 to 100.9%.

### RESULTS

Hemoglobin values are shown in table 2; there was no correlation between this compound and the ascorbic acid content of plasma or whole blood. The difference in hemoglobin value for the two sexes was statistically significant.

TABLE 2

*Mean ascorbic acid and hemoglobin values of blood of rats grouped according to body weight and sex. Range and standard error are included.*

BODY WEIGHT	HEMOGLOBIN	ASCORBIC ACID	
		Plasma	Whole blood
<i>gm</i>	<i>gm/100 ml</i>	<i>mg/100 ml</i>	<i>mg/100 ml</i>
<b>Males</b>			
250-299 (23)	13.6 $\pm$ 0.14 (12.0-14.7)	0.84 $\pm$ 0.052 (0.33-1.36)	0.70 $\pm$ 0.026 (0.49-0.91)
300-450 (27)	13.8 $\pm$ 0.13 (12.8-16.1)	0.90 $\pm$ 0.026 (0.62-1.21)	0.74 $\pm$ 0.022 (0.55-0.99)
Mean (50)	13.7 $\pm$ 0.10	0.87 $\pm$ 0.028	0.72 $\pm$ 0.017
<b>Females</b>			
200-249 (26)	13.0 $\pm$ 0.11 (11.9-14.1)	0.33 $\pm$ 0.029 (0.01-0.63)	0.34 $\pm$ 0.011 (0.23-0.46)
250-350 (25)	13.0 $\pm$ 0.21 (11.2-14.8)	0.33 $\pm$ 0.024 (0.09-0.61)	0.36 $\pm$ 0.019 (0.23-0.65)
Mean (51)	13.0 $\pm$ 0.12	0.33 $\pm$ 0.019	0.35 $\pm$ 0.011

The values for ascorbic acid in whole blood and plasma for both sexes are given in table 2. The males had a mean value of  $0.87 \pm .028$  mg per 100 ml of plasma, with a range of 0.33 to 1.36 mg; the mean value for whole blood was  $0.72 \pm .017$  mg, with a range of 0.49 to 0.99 mg. The corresponding values for females were  $0.33 \pm 0.019$  mg with a range of 0.01 to 0.63 mg for plasma, and  $0.35 \pm 0.011$  mg with a range of 0.23 to 0.65 mg for whole blood.

There was no correlation for either sex between the ascorbic acid in the plasma or whole blood and the age or weight of the adult animals.

There was a highly significant difference between the two sexes in ascorbic acid content of both whole blood ( $0.37 \pm .020$  mg) and plasma ( $0.54 \pm .033$  mg). The reason for this difference is not apparent. It is possible that the sexes have equal ability to synthesize ascorbic acid but that utilization is greater on the part of the female and thus brings about lower blood values. Sutton et al. ('42) reported ascorbic acid values of 0.588 mg to 0.895 mg per 100 mg of plasma for male rats receiving varying levels of vitamin A as supplements to the diet. No plasma values were given for females but there was found to be a sex difference in the excretion of ascorbic acid. Female rats excreted only about half as much ascorbic acid as did the males.

An interesting relationship was shown between the ascorbic acid content of the plasma and whole blood (table 3). At the lower levels of plasma ascorbic acid, 0.01 to 0.60 mg for males and 0.01 to 0.30 mg for females, the values for ascorbic acid per 100 ml of whole blood were higher than the plasma values, but from that point, at any plasma level the value for whole blood was lower than the plasma value.

Butler and Cushman ('40) using different methods of analysis have reported that at low levels, or even when the plasma ascorbic acid has become zero there are still measurable amounts of ascorbic acid in the whole blood. In this connection it is of interest to note that in the present study the males, whose mean whole blood ascorbic acid level was 0.83 mg, showed a highly significant difference of  $0.15 \pm 0.032$  mg between whole blood and plasma levels. In contrast, the females, with the definitely lower mean whole blood value of 0.35 mg, did not have a significantly different plasma level. These data support the view that ascorbic acid enters the red cells with difficulty and tends to remain in the cells when the plasma level falls.



If it can be assumed that the blood plasma levels for all mammals are similar (table 1) and that the values obtained for mature rats which synthesize their ascorbic acid are indicative also of normal levels for humans then plasma values currently reported as desirable for humans are too high, at least for females.

The data of the present report also indicate the desirability of studying the sexes separately in ascorbic acid investigations.

TABLE 3

*Difference between whole blood and plasma ascorbic acid at various plasma levels for albino rats.*

PLASMA ASCORBIC ACID	MALE		FEMALE		MALE AND FEMALE	
	Number	Whole blood minus plasma (average)	Number	Whole blood minus plasma (average)	Number	Whole blood minus plasma (average)
<i>mg/100 ml</i>		<i>mg</i>		<i>mg</i>		<i>mg</i>
0.01-0.09			3	+ .243	3	+ .243
0.10-0.19			5	+ .140	5	+ .140
0.20-0.29			10	+ .054	10	+ .054
0.30-0.39	2	+ .215	17	— .003	19	+ .020
0.40-0.49			12	— .050	12	— .050
0.50-0.59	1	+ .020	2	— .125	3	— .079
0.60-0.69	4	— .050	2	— .100	6	— .066
0.70-0.79	8	— .100			8	— .100
0.80-0.89	13	— .128			13	— .128
0.90-0.99	8	— .195			8	— .195
1.00-1.09	10	— .218			10	— .218
1.10-1.19	1	— .320			1	— .320
1.20-1.29	2	— .440			2	— .440
1.30-1.39	1	— .450			1	— .450

## SUMMARY

Mature, normal rats, 50 males and 51 females, were sacrificed for determination of the ascorbic acid content of the plasma and whole blood, and of hemoglobin values.

The ascorbic acid content of the plasma of males was  $0.87 \pm .028$  mg, and of females was  $0.33 \pm .019$  mg per 100 ml; the difference was highly significant.

The ascorbic acid content of the whole blood of males was  $0.72 \pm .017$  mg and of females was  $0.35 \pm .011$  mg per 100 ml; the difference again was highly significant.

There was a significant difference in hemoglobin values for the two sexes,  $13.7 \pm 0.10$  gm per 100 ml for males and  $13.0 \pm 0.12$  gm for females. There was no correlation between the hemoglobin and the ascorbic acid content of the blood nor between either of these substances and the weight or age of the adult animals.

At low levels of plasma ascorbic acid the corresponding values for ascorbic acid in whole blood were higher; at plasma levels of 0.59 mg for males and 0.30 mg for females the order of the values was reversed.

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# THE RELATIVE EFFICIENCY OF DIFFERENT FORMS OF INTRAVENOUSLY ADMINISTERED NITROGEN ON NITROGEN BALANCE AND AMINO ACID EXCRETION<sup>1</sup>

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## FIVE FIGURES

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Methods for determining the relative nutritive values of proteins have been in use for many years. These involve principally measurement of the rate of growth, gain in weight per gram of nitrogen ingested, nitrogen exchange and balance, reproduction, and plasma protein and hemoglobin regeneration (Mitchell and Hamilton, '29). The recent availability of protein hydrolysates and crystalline amino acids suitable for parenteral injection raises the question of the relative values of such substances for nitrogen retention when they are given by vein. Since the report of Henriques and Andersen in 1913 it has been repeatedly shown that protein hydrolysates can be utilized by the body when given intravenously (Elman and Weiner, '39; Shohl et al., '39; Farr and MacFayden, '39; Elman, '40). However, the efficiency with which such materials effect nitrogen retention when given by vein is not known. Such factors as the unnatural isomers, deleterious changes during manufacture or processing, varying rates of injection and the renal excretion of amino acids and

<sup>1</sup> Presented before the Society for Pediatric Research, Atlantic City, September 25-26, 1944.

peptides, may conceivably interfere with the utilization of forms of nitrogen which are satisfactory when orally ingested.

A limited number of observations comparing oral and parenteral administration or comparing various nitrogenous substances when parenterally administered have been made. Elman et al. ('42), showed that an enzymic casein hydrolysate was as effective in plasma protein regeneration when intravenously administered as when it was given orally. Cox and Mueller ('44 b) compared enzymic hydrolysates of three proteins, casein, lactalbumin and plasma protein, when both intravenously and orally administered. Madden et al. ('44) compared mixtures of crystalline amino acids with various protein hydrolysates administered parenterally as well as orally to anemic and hypoproteinemic dogs, and a recent report from the same laboratory indicates that intravenous administration is not as effective for plasma protein production as the oral route (Madden et al., '45 a). Albright ('45) has found oral or intravenous administration of a casein hydrolysate equally effective in maintaining nitrogen balance.

To study the efficiency of nitrogen retention, three dogs were depleted of their nitrogen stores by feeding a diet adequate in calories but low in protein for a protracted period. Subsequently, during periods of not less than 10 days each an amount of nitrogen calculated to just equal the average daily nitrogen loss for the last 17 days of the depletion period was injected intravenously. With this low level it was expected that too little nitrogen would be retained to affect the nitrogen deficit, and that the dog would remain suitable for comparative assay purposes. In actual fact there was usually nitrogen equilibrium or a slight nitrogen loss during the first assay period so that the nitrogen need of the animal was unchanged.

Three sources of nitrogen were employed: a pancreatic hydrolysate of casein<sup>2</sup>; a mixture of ten essential amino acids and glycine (Mixture V<sub>u</sub> of Madden<sup>3</sup>); and a mixture of ten essential and ten unessential amino acids which we calculated

<sup>2</sup> Amigen.

<sup>3</sup> The composition of this mixture was given to us by Dr. Madden.

as representing the approximate composition of casein (referred to as Mixture XII). Each was administered intravenously for a total of 10 days in 6 to 10% solution (as determined by solubility) at the same nitrogen level. When studies on these low levels had been completed, it seemed also desirable to compare retentions at higher nitrogen levels. Accordingly the minimal nitrogen level was doubled and the nitrogen balances for periods of 10 days again compared. The casein hydrolysate and mixture V<sub>a</sub> were then compared at nitrogen injection levels which were four and eight times the minimum calculated for equilibrium.

At the lowest level of nitrogen the mixture of ten essential amino acids and glycine was most efficient in promoting retention; at the doubled level this mixture and the hydrolysate were about equally effective, and at the quadrupled and octupled levels, the hydrolysate effected much larger retention than the crystalline amino acid mixture. The mixture of amino acids calculated to represent the composition of casein was considerably less efficient in retention than the other two preparations.

#### EXPERIMENTAL

The three dogs selected for this study had been previously maintained in metabolism cages on various levels of nitrogen for about 100 days. During a portion of the time they had received intravenous injections of amino acid mixtures and the casein hydrolysate in a study unrelated to the present one. The basal diet consisted of a mixture of the Weech-Goettsch ('38) low protein diet and a commercial preparation known as Gaines' dog food. It was supplemented by the daily administration to each dog of 3 mg thiamine, 6 mg riboflavin, 30 mg niacin and 5 drops *Oleum Percomorphum* since in earlier studies signs of vitamin B deficiency had been observed when the dogs subsisted for protracted periods on the Weech-Goettsch diet. The essential nutritive histories are as follows:

Dog 1 ("Nig"), a male mongrel hound, received 0.25 gm nitrogen and 40-70 cal. per kg body weight daily in his basal

diet for 110 days; and at intervals, intravenous injections of various forms of nitrogen. At the end of this period he weighed 57 pounds and was in good nutritive condition. Dog no. 2 ("Police"), a male police dog, weighed 49 pounds when brought from the kennels and placed on metabolism. During the preliminary 110-day period he was fed 0.44 gm of nitrogen and 40 cal. per kg body weight, and also received certain intravenous injections of nitrogen. He weighed 44 pounds at the end of the 110-day period, and was in excellent condition. Dog no. 3 ("Part Police") a male mongrel police, weighed 42.5 pounds in the kennels when his food was reduced to near starvation — 8 cal. and 0.1 gm nitrogen per kilo, daily for 15 days. For the next 27 days he was fed 43 cal. and 0.12 gm of nitrogen per kilogram. During these 42 days he lost 10.5 pounds and at the end was in very poor nutritive condition. Supplementation with various levels of intravenously administered nitrogen during the next 63 days restored his weight to 36.5 pounds, at which time the present study was begun.

Since the early report of Cowgill ('28) it has been customary to feed dogs 80 cal. per kg body weight. This amount is based on the ad libitum food consumption of dogs allowed a moderate amount of exercise. Since Benedict ('38) has shown that the basal heat requirement of dogs is approximately 35 cal. per kg, it has been our custom to supply 50 cal. per kg to dogs confined in metabolism cages. In our experience this has been a satisfactory energy level. Collections of urine (preserved with thymol) and feces were made daily, and combined to make periods of 3 or 4 days. The dogs were not catheterized.

*Depletion period.* At the start of the study, the three dogs were fed a mixture of the two diets mentioned above calculated to supply progressively smaller amounts of nitrogen and a constant caloric intake. The minimum daily nitrogen intake was .04 gm per kilo for from 17 to 24 days. The depletion period lasted 56 days. The average daily loss during the last 17 days was .099, .057 and .091 gm nitrogen per kg body weight

for Dogs 1, 2 and 3, respectively. They lost, respectively, a total of 130, 82 and 57 gm of nitrogen, and in body weight 3.5, 1.6 and 2.5 kg, during the whole depletion period.

*Supplementation materials.* The particular lot of casein hydrolysate (10,014-15) employed in this work was not as adequate for nutrition as most manufactured batches (Mueller et al., '40). The total nitrogen was 12.4%, the amino nitrogen 7.7% and the amino nitrogen after acid hydrolysis, 10.5%. The average gain in weight per day of 10 litter-mate rats maintained for 8 weeks on 3 different levels of this lot as the

TABLE 1  
*Per cent composition of amino acid mixtures.*

	Mixture V <sub>a</sub> <sup>1</sup>	Mixture XII <sup>2</sup>
dl-threonine	7.0	2.0
dl-valine	11.0	12.6
dl-leucine	18.0	7.1
dl-isoleucine	12.0	6.3
l(+) lysine HCl	12.0	9.1
dl-tryptophane <sup>3</sup>	4.0	1.8
dl-phenylalanine	12.0	6.2
dl-methionine	6.0	2.8
l(+) histidine HCl	4.0	2.7
l(+) arginine HCl	7.0	5.0
Glycine	7.0	2.4
dl-alanine		3.0
dl-norleucine		2.0
l(—) proline		6.3
l(—) hydroxyproline		1.6
l(+) glutamic acid		17.4
dl-aspartic acid		3.2
dl-serine		2.4
l-tyrosine		5.1
l-cystine		1.0

<sup>1</sup> Composition kindly furnished by Dr. S. C. Madden.

<sup>2</sup> The analytical values which we chose for this mixture were too low in threonine and leucine, and should have been doubled since the unnatural forms are not available for growth. This quantitative deficiency accounts for the observed lower nitrogen retention. NaHCO<sub>3</sub>, 9.2 gm per 100 gm of the amino acid mixture was added just before making the solution.

<sup>3</sup> Mixture V<sub>a</sub> was designed to supply 4.0% l-tryptophane. Since we had only the dl-form available, this was used at the same level as there is evidence that the unnatural isomer is utilized; and even if it is not, 2% of the natural isomer is probably adequate.



sole source of nitrogen was: 1.2% nitrogen, 1.14 gm daily gain, 1.7% nitrogen, 1.45 gm gain; 2.4% nitrogen, 1.67 gm gain. It was made in 10% solution, sterilized by Seitz filtration and given intravenously by slow drip. The composition of the two mixtures of crystalline amino acids is given in table 1. Limited by solubilities, these were made in 7% solution and sterilized by autoclaving. Mixture XII deposited crystals, presumably tyrosine, on standing. These were filtered off, and a nitrogen determination made on the filtered solution in order to calculate the amount to be injected.

TABLE 2

*Comparative amounts of essential amino acids supplied by the three supplements.*

AMINO ACID	GM/DAY SUPPLIED ONE DOG BY LOWEST AMIGEN LEVEL	MULTIPLES OF AMIGEN SUPPLIED BY	
		Mixture XII	Mixture V <sub>u</sub>
Threonine	0.57	0.66	2.16
Valine	0.97	2.44	1.98
Leucine	1.76	0.76	1.79
Isoleucine	0.90	1.32	2.34
Lysine	0.97	1.76	1.82
Tryptophane	0.26	1.32	2.73
Phenylalanine	0.75	1.55	2.79
Methionine	0.49	1.06	2.12
Histidine	0.37	1.38	1.91
Arginine	0.62	1.51	1.97
Gm essential amino acids daily	7.65	10.43	16.23
Gm mixture daily	18.29	18.79	17.45

These three materials necessarily differed in their content of the essential amino acids. As an approximation only, the amounts of the essential amino acids supplied by the hydrolysate, by Mixture V<sub>u</sub> and by Mixture XII are compared in table 2. The amount of these acids present in the hydrolysate was determined by calculation from the amino acid composition of casein and pancreas as given by Block and Bolling ('45) and was used as the basis of comparison. The values (in grams of the hydrolysate) are based on the weight of

material supplied to one dog, and the values in the other two columns express the relative amount of each amino acid contained in the mixture when it was given at the same nitrogen level. This comparison shows Mixture XII (since it was based on early values for the composition of casein) to contain less threonine and leucine than the calculated amount in the hydrolysate. Since the unnatural isomers of certain amino acids are probably not used in metabolism (Gilman, '43), the mixture may also be relatively low in the active form of isoleucine. All of the amino acids contained in the enzymic hydrolysate are probably in the active form. Mixture V<sub>u</sub> contained about twice as much of each essential amino acid as contained in the hydrolysate, and this quantity would allow for the unnatural enantiomorphs.

This comparison must be interpreted with caution, because the composition of the hydrolysate is calculated, and no correction for the optical forms of the pure acids is made. An assay of a sample of the hydrolysate using available techniques<sup>4</sup> showed values lower than those calculated for certain acids, and higher values for others. The specific values, calculated and determined are, respectively: leucine 9.6, 8.0; methionine 2.7, 2.0; arginine 3.4, 4.9; phenylalanine 4.1, 4.0; isoleucine 4.9, 6.0; valine 5.3, 5.6; tryptophane 1.0, 0.6%.

*Administration.* All infusions were given by slow intravenous drip in the radial veins while the dogs were suspended in a Pavlov frame with legs dependent. The rate was adjusted to the tolerance of the dog, so as to prevent nausea and vomiting.

## RESULTS

The data are presented graphically in figures 1, 2 and 3. The values for caloric intake and nitrogen intake are plotted per kg body weight. Nitrogen balance, urinary and fecal nitrogen, weight, plasma albumin and total plasma protein and the hematocrit values are plotted in absolute quantities.

<sup>4</sup> Values determined for us by William T. Thompson Co., Los Angeles, using a microbiological assay procedure developed by Shankman et al. ('43)

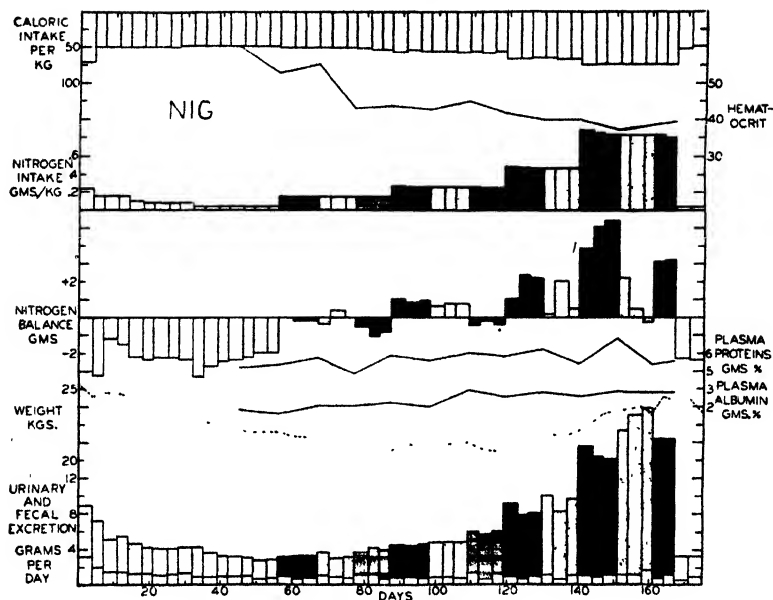


Figure 1



Figure 2

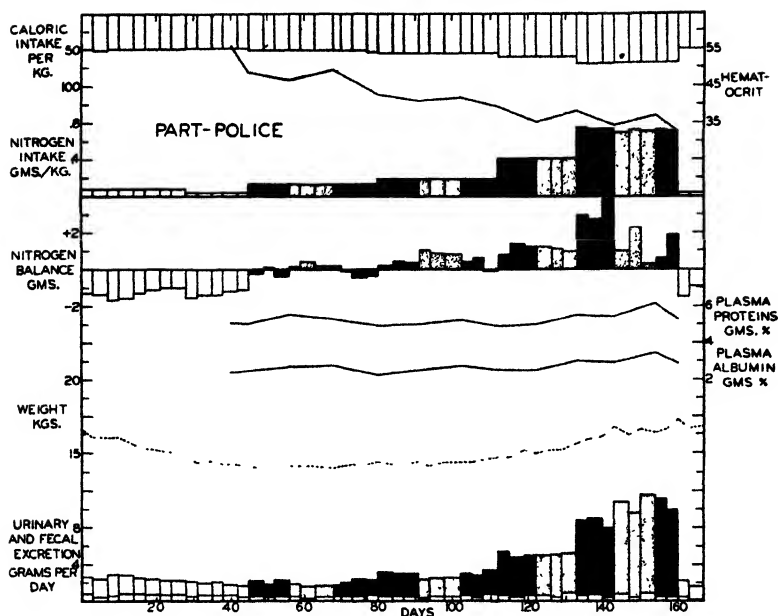


Fig. 3 Graphic record of Dog 3, constructed as described for figure 1. With this dog the nitrogen retention with mixture XII equalled that with the hydrolysate.

*Nitrogen balance.* At the lowest level, each of the 3 dogs retained the  $V_u$  mixture of amino acids slightly more efficiently than the casein hydrolysate. Dogs 2 and 3 showed the most pronounced difference in retention while Dog 1 showed but little. At the doubled levels the average nitrogen balances for Dogs 2 and 3 again showed a higher retention with Mixture  $V_u$ , although Dog 1 retained the casein hydrolysate most efficiently at this level. At the quadrupled levels the situation

Fig. 1 Graphic record of Dog 1 during protein depletion and the subsequent intravenous injection of 3 sources of nitrogen at four intake levels. The injections of enzymic hydrolysate (Amigen) are indicated by the solid black columns; the ten crystalline essential amino acids and glycine ( $V_u$ ) by the dotted columns, and a mixture of ten essential and ten unessential amino acids (XII) by the horizontally-ruled columns. Each indicated period covers 3 or 4 days, and each supplement was administered for at least 10 days. Urinary and fecal nitrogen excretion are separately indicated.

Fig. 2 Graphic record of Dog 2, constructed as described for figure 1. The nitrogen loss of this dog during depletion was considerably less than that of the other two and correspondingly less nitrogen was required to establish equilibrium.

was reversed, and all three dogs retained more nitrogen from the casein hydrolysate than from Mixture  $V_u$ . The difference in retention in favor of the casein hydrolysate, was even more striking when the injection level was octupled. The dogs retained 4.8, 3.1 and 2.1 gm of nitrogen daily when the hydrolysate was injected, and 1.0, 1.1 and 0.8 gm with Mixture  $V_u$ .

Nitrogen retention with Mixture XII did not equal that of the casein hydrolysate or Mixture  $V_u$ . It was injected at only

TABLE 3  
*Summarized nitrogen balances. Gm per day.*

	"NIG"	"PART POLICE"	"POLICE"	AVERAGE FOR 3 DOGS
<i>Casein hydroly- sate</i>				
Low level	- 0.11	- 0.19	- 0.27	- 0.19
× 2	+ 0.91	+ 0.31	+ 0.59	+ 0.60
× 4	+ 1.82	+ 1.14	+ 1.49	+ 1.48
× 8	+ 4.78	+ 3.14	+ 2.06	+ 3.33
× 8	+ 3.20	+ 1.29	+ 1.56	+ 2.02
<i>Mixture <math>V_u</math></i>				
Low level	- 0.04	+ 0.28	+ 0.25	+ 0.16
× 2	+ 0.72	+ 0.87	+ 0.73	+ 0.78
× 4	+ 1.03	+ 1.08	+ 0.79	+ 0.97
× 8	+ 0.81	+ 1.09	+ 1.21	+ 1.04
<i>Mixture XII</i>				
Low level	- 0.75	- 0.22	- 0.06	- 0.34
× 2	- 0.33	+ 0.27	+ 0.06	+ 0.00

two levels since it had to be given much more slowly than the other two preparations in order to avoid nausea and vomiting.

For careful comparison, the summarized nitrogen balance data are given in table 3 for the individual dogs. The 3 dogs behaved similarly to all three supplements, so that the average values are representative. These show that at the two lowest levels of supplementation Mixture  $V_u$  was more effective for retention than the casein hydrolysate, but that at the two higher levels the situation was reversed. To determine whether the less efficient retention of Mixture  $V_u$  at the

higher levels was due to the sequence of supplementation, or perhaps to the decrease in nitrogen need after so much retention on the casein hydrolysate, a second period using the octupled level of the hydrolysate was given. It is evident from the charts and from table 3 that retention was still high, and was considerably greater than that with the amino acid ( $V_u$ ) mixture.

*Weight.* The close parallelism between weight and nitrogen balance is best seen from figure 1. Weight declined during depletion, and was stationary when minimal levels of nitrogen were given, and increased slowly when the nitrogen level reached adequacy.

*Plasma albumin.* As shown in figure 1, the plasma albumin value rose from 1.6 gm per cent at the end of the depletion period to 2.8 gm per cent at the end of intravenous supplementation. This change does not measure the rapidity of albumin regeneration with parenterally administered nitrogen since the level given during most of the experimental period was inadequate for maximal protein synthesis. The other two dogs (figures 2 and 3) also showed changes in plasma albumin of about the same magnitude.

*Hematocrit.* The hematocrit values for all three dogs declined during the experimental period. We have no explanation. The experimental diet contained a sufficient amount of iron.

*Caloric intake.* Except for the caloric value of the nitrogen given by vein the caloric intake was maintained at 50 cal. per kilo body weight. At the octupled level, the supplement increased the intake of Dogs 1 and 3 by about 20 cal. per kg and Dog No. 2 about 10 cal. At the lower nitrogen levels the calories supplied by the supplements did not significantly affect the caloric intake; nor did this increase at the higher level affect the findings.

*Excretion.* As indicated at the bottom of all three figures the fecal excretion of nitrogen was not modified by the level of intravenous supplementation. The urinary excretion

responded promptly to the injection level, and determined the magnitude of the nitrogen balance.

*Excretion of amino acids and peptides.* The excretion of amino acids and peptides during all of the periods has been measured. The ninhydrin procedure of Van Slyke et al. ('43) was used to measure the amino nitrogen in the urine. The urine was subsequently hydrolyzed by boiling 10 ml with 10 ml of concentrated hydrochloric acid for 12 hours and the amino nitrogen value re-determined. The difference was attributed to peptides.

Figures 4 and 5 show the magnitude of amino acid excretion as measured by the amino nitrogen. It is evident, when the same levels of supplementation are compared, that the crystalline mixtures resulted in a much greater excretion of amino acids than did the hydrolysate. In all probability this is due to the excretion of unnatural forms of the amino acids, but rapid filtration of amino acids through the kidney glomeruli may contribute to the total value. It is not due to differences in rate of injection. The values recorded above the curves indicate the average number of minutes required daily for the injection of the particular supplement. In figure 4, for example, it required an average of 183 minutes daily to inject the quadrupled level of the hydrolysate, and 277 minutes to inject the same level of crystalline acids ( $V_u$ ). At both

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Fig. 4 Urinary amino nitrogen excretions of Dog 1. The periods at the extreme left and right of the chart indicate the excretion when no infusions were administered. The solid black columns are periods during which the enzymic hydrolysate (Amigen) was injected; the dotted columns are periods during which the ten crystalline essential amino acids and glycine ( $V_u$ ) were injected, and the ruled columns are periods during which the mixture of ten essential and ten unessential amino acids (XII) was given. The nitrogen injection level is indicated below the columns; thus " $4 \times V_u$ " indicates that the  $V_u$  mixture was given at 4 times the minimal level. Each individual column represents the average daily 24-hour excretion for periods of 3 or 4 days. Each supplement was given for not less than 10 days. The numbers at the top of the columns are the average minutes required to administer the infusion.

Fig. 5 Urinary amino nitrogen excretions of Dogs 2 and 3. Description of the chart is given under figure 4. The amino nitrogen excreted after the injection of the crystalline mixtures is always greater than following the hydrolysate.

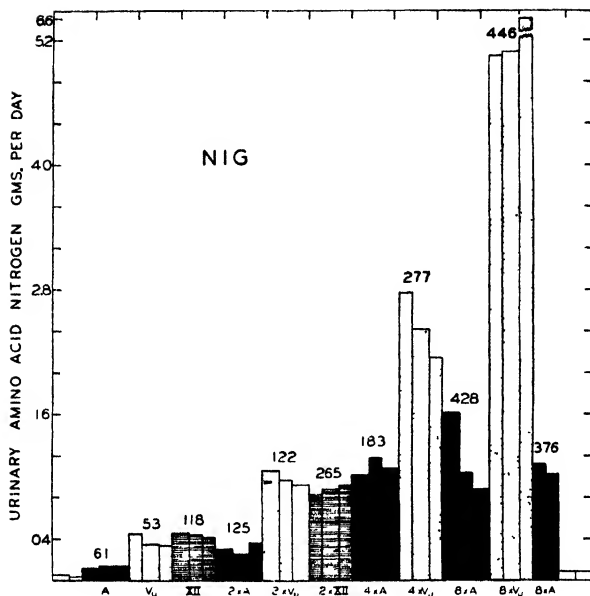


Figure 4

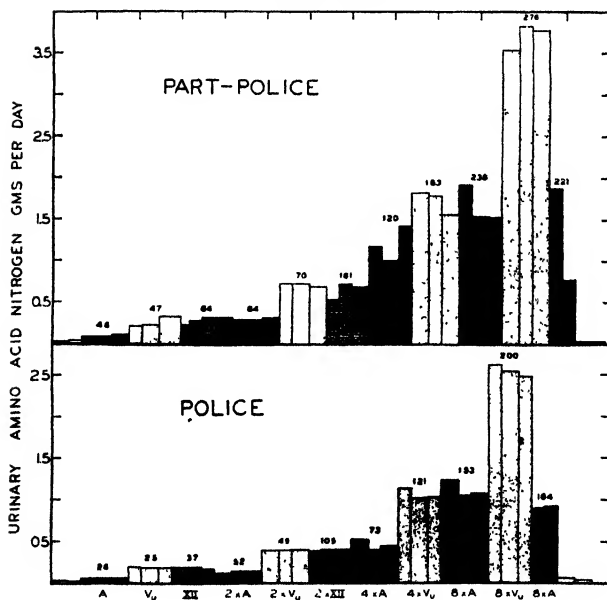


Figure 5



of the high injection levels, the crystalline acids were injected more slowly than the hydrolysate.<sup>5</sup>

The amino nitrogen of all urines from injections of the hydrolysate increased after acid hydrolysis, indicating that a certain amount of the injected peptides had been excreted. Table 4 shows the percentage of the injected nitrogen which

TABLE 4

*Per cent of injected nitrogen excreted as amino and peptide nitrogen.*

	INJECTION TIME MINUTES DAILY	AMINO ACID NITROGEN	PEPTIDE NITROGEN	TOTAL NITROGEN IN BOTH FORMS
Minimum level				
Amigen	44	3.55	9.26	12.81
Mixture V <sub>u</sub>	42	15.37	1.01	16.38
2 × minimum level				
Amigen	87	6.82	11.22	18.04
Mixture V <sub>u</sub>	80	20.40		20.40
4 × minimum level				
Amigen	125	14.09	11.78	25.87
Mixture V <sub>u</sub>	187	27.08	0.59	27.67
8 × minimum level				
Amigen	273	10.65	12.08	22.73
Mixture V <sub>u</sub>	307	31.18	0.92	32.10

was excreted as amino acid nitrogen, as peptide nitrogen, and in total when the values from all 3 dogs were averaged.

The table shows that the sum of both forms excreted when the different supplements were given is nearly the same. The crystalline acids resulted in the excretion of only slightly more total nitrogen than the hydrolysate, and this was significant only at the highest level.

<sup>5</sup> This rate of injection was determined by the tendency of the dogs to vomit. At the octupled level, for example, the hydrolysate was injected for 50 days and there were 5 days on which the dogs vomited, but the same level of Mixture V<sub>u</sub> resulted in an episode of vomiting on 21 of the 30 days on which it was injected. This accounted for the slower average rate of injection for this material.

By comparing the amount of peptide nitrogen injected with the peptide nitrogen excreted, it was determined that 40 to 50% of the injected peptide nitrogen appeared in the urine. This fact needs further study since we have found that pure crystalline dipeptides are utilized when given intravenously. The average chain length of the peptides in the casein hydrolysate approximated 3.4 when the method of calculating the chain length as described by Stein et al. ('44) was used.

#### DISCUSSION

This comparative study of nitrogen retention when an enzymic hydrolysate and mixtures of amino acids are given intravenously indicates that the essential amino acid composition of a substance will not necessarily determine its value. The administration of 7.65 gm of essential amino acids as the hydrolysate was almost as effective as 16.23 gm of the crystalline essential acids (table 2). At higher levels, but at the same ratio of intake the smaller quantity from the hydrolysate was much more effective in accomplishing nitrogen retention. This difference may be partially explained by the presence of unnatural isomers in the synthetic amino acids, and partially by rapid excretion. But it also indicates to us that the body requires certain amino acids which are not classified as essential. The formation of new protein requires the presence of a number of unessential amino acids and if they are not specifically supplied in the diet, the essential acids will be utilized to form them. It is thus conceivable that if essential amino acids constitute the principal nitrogen of the diet, that more would be required than if certain quantities of unessential amino acids were also given.

The observations further indicate that the hydrolysate can probably be modified by the addition of amino acids to the end that nitrogen retention on minimal levels can be improved. Such studies are now in progress.

Nausea and vomiting accompany the rapid administration of protein hydrolysates and amino acids (Cox and Mueller, '44 a, b; Madden et al., '45 a). Our present observations with

three sources of amino acid nitrogen indicate that the two crystalline mixtures were more prone to cause such disturbances than an enzymic hydrolysate. No satisfactory explanation for the tendency to emesis caused by parenteral administration of amino acids has been found. It is worthy of note that Mixture V<sub>a</sub> contained no glutamic (Madden et al., '45 b) or aspartic acid (Madden et al., '45 a) and did contain glycine (Madden et al., '44), and that its tendency to cause vomiting was greater than that of the hydrolysate. The level of amino nitrogen in the blood has been suggested as the factor provoking emesis but this is far from a satisfactory index (Cox and Mueller, '43). Madden's observations indicate that certain mixtures of amino acids provoke less vomiting than others but in our experience dogs vary widely in their reaction to a given mixture on successive days so that it is impossible to incriminate any one essential amino acid (Cox and Mueller, '44 a).

#### CONCLUSION

An enzymic casein hydrolysate and two mixtures of crystalline amino acids have been compared for their ability to promote nitrogen retention in protein depleted dogs when given intravenously at varying levels of nitrogen intake.

At levels just sufficient to establish nitrogen equilibrium the mixture of ten essential amino acids and glycine (V<sub>a</sub>) was slightly more effective in promoting nitrogen retention than the other two substances. At higher levels of intake the hydrolysate was considerably more effective than the V<sub>a</sub> mixture.

Urinary excretion of amino acid nitrogen was always greater with the crystalline mixtures than with the hydrolysate. Conversely, more peptide nitrogen appeared in the urine when the hydrolysate was given. The sums of nitrogen lost in both forms were approximately equal although the crystalline mixture was given more slowly than the hydrolysate in order to avoid emesis.

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# THE INFLUENCE OF SOURCES OF PHOSPHORUS ON THE RELATIVE EFFICIENCY OF VITAMIN D<sub>3</sub> AND COD LIVER OIL IN PROMOTING CALCIFICATION IN POULTS <sup>1</sup>

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## ONE FIGURE

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The work of Fritz, Hooper and Moore ('45), which has been essentially confirmed by Evans and Brant ('45), would indicate that the discrepancies reported in the literature pertaining to the vitamin D requirements of poults, can be explained in part by the wide variation in the levels of either calcium or phosphorus that have been used in the test diets. Previously Bird ('44) and Boucher ('44) had indicated that this might be a possible explanation.

That vitamin D<sub>3</sub> is approximately two times as efficient as fish liver oil in promoting calcification in the poult, chick unit for chick unit, has been reported by Boucher ('44) and Bird ('44). Fritz et al. ('45) observed the same trend but quantitatively the differences noted by them were much less marked than those reported by either Boucher or Bird. The "Borden Diet" employed by Fritz and co-workers analyzed 1.51% calcium and 1.0% phosphorus. The phosphorus content of the "Borden Diet" was greater than that of the diets used by either Bird or Boucher and in addition must have contained

<sup>1</sup> Supported in part by a grant from the Yantic Grain and Products Company, Norwich, Connecticut.

a greater amount of readily available phosphorus. This might explain why extreme differences in response were not observed by Fritz and his co-workers. It should be noted that the diet employed by Boucher contained a relatively small amount of non-phytin phosphorus. We have estimated that his ration contained less than 0.2% of non-phytin phosphorus. The composition of Bird's diet, while similar to that of Boucher's but containing a greater per cent of phosphorus (0.82%), may also have been low in readily available phosphorus (*vide infra*).

It is conceivable that the results obtained by Boucher and others and interpreted as a species specificity, could be explained on the basis of the availability of the phosphorus as well as the absolute level of this mineral in the diets. Moreover, the wide differences of opinion in regard to the vitamin D requirements of the poult, may, in part at least, have originated because of differences in readily available phosphorus in the rations. In Boucher's work as well as that of Bird, the vitamin D carriers were first assayed on the A.O.A.C. diet with chicks, which is relatively high in inorganic phosphorus. The vitamin supplements were then incorporated in turkey rations relatively low in inorganic phosphorus considering the greater known mineral requirements of the poult. It should be emphasized that poults were not fed the A.O.A.C. diet nor were chicks fed the poult diets. However, from pilot experiments in progress at this station, it should be stated that there is some indication that the A.O.A.C. chick diet when fed to poults may not contain a sufficient amount of calcium and of readily available phosphorus to assay adequately vitamin D carriers.

It occurred to one of us (H.M.S.) that the source of vitamin D might account for differences observed in bone ash of chicks on a diet high in phytin phosphorus. This idea was developed by Singsen and Mitchell ('45) who have shown that activated animal sterols (Delsterol) are 1.34 times as effective as a pure cod liver oil in promoting calcification in the chick when the chick must rely upon phytin phosphorus to meet its require-

ments. That poult subjected to a similar experimental design also exhibit this differential in response is indicated in the data presented here.

The objectives of this work were: (1) to determine the response of turkey poult to vitamin D from activated 7-dehydrocholesterol and a pure cod liver oil when incorporated in a simplified diet in which all of the phosphorus was readily available and (2) to measure the effectiveness of these two sources of vitamin D in promoting the utilization of the phosphorus of phytin when the poult is forced to rely upon phytin to meet its phosphorus requirements.

#### EXPERIMENTAL

The low phosphorus basal mixture consisted of white table corn meal <sup>2</sup> 67.3, casein 12.0, gelatin 3.0, primary grown yeast 4.0, bagasse 4.0, liver meal 3.0, wheat germ oil 1.0, CaCO<sub>3</sub> 2.23, KCl 0.5, and salt mixture 1.6.<sup>3</sup> The vitamin supplement per 100 pounds of the ration consisted of calcium pantothenate 635 mg, pyridoxine 227 mg, riboflavin 227 mg, vitamin K 18 mg, thiamine chloride 95 mg, nicotinic acid 95 mg, inositol 227 mg, para amino benzoic acid 227 mg, choline chloride 45.4 gm and vitamin A 800,000 I.U. On analysis this basal mixture was shown to contain 1.21% calcium and 0.23% phosphorus.

Two experiments were conducted in which the vitamin D from a pure cod liver oil was compared with activated 7-dehydrocholesterol, the principle dietary variation being the source of phosphorus used.

In experiment 1, NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O (1.37%) was added to the basal diet to bring the total phosphorus level to 0.61%, all of which was readily available. In experiment 2, calcium-magnesium phytate (2.09%) raised the total phosphorus level of the basal diet to 0.55%. Phytin phosphorus by analysis,

<sup>2</sup> A de-germed, de-braned product especially prepared for table use and containing only 0.07% phosphorus of which less than 0.01% is phytin.

<sup>3</sup> 1000 gm NaCl, 10 gm KI, 150 gm MnSO<sub>4</sub>·H<sub>2</sub>O, 50 gm FeSO<sub>4</sub>·x H<sub>2</sub>O, 136 gm MgSO<sub>4</sub>, 5 gm ZnSO<sub>4</sub>, 5 gm CaSO<sub>4</sub>·5 H<sub>2</sub>O, 200 gm NaSiO<sub>3</sub>, 6 H<sub>2</sub>O, 50 gm Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>, 2 gm Co(CH<sub>3</sub>COO)<sub>2</sub>·4 H<sub>2</sub>O.



accounted for 55.4% of the total phosphorus in the diet. It was the original intent to have the level of phosphorus identical in both rations.

The pure cod liver oil and the activated 7-dehydrocholesterol were assayed in our laboratory by the A.O.A.C. procedure and incorporated in the diets on a chick unitage basis.<sup>4</sup> Twenty bronze day-old poults, all originating from the same breeding stock maintained by the station were placed in each experimental lot. They were confined to electric brooding compartments located in a room where all daylight was excluded. At the conclusion of the experimental period all surviving poults were weighed and then sacrificed for the removal of the left tibiae. The bones were cleaned, extracted in alcohol for 48 hours, ether for 24 hours and then ashed individually. Because of the extreme degree of rachitis manifested by all lots fed the phytin phosphorus diet this experiment was terminated at 21 days. The results are presented in table 1.

#### DISCUSSION

An examination of the data presented in table 1 (Expt. 1) shows clearly that 7-dehydrocholesterol when incorporated in the inorganic phosphorus diet was no more efficient in promoting calcification than was cod liver oil when the two sources of vitamin D were fed on an equal chick unitage basis. At the 80 unit level the 7-dehydrocholesterol did give a higher percentage of bone ash than did the cod liver oil. However, this is thought to be due to chance since all other levels in both series are essentially equal.

Where the design of the experiment is such that the poult is forced to rely upon phytin phosphorus for bone formation (Expt. 2), the greater efficiency of vitamin D<sub>3</sub>

<sup>4</sup> The 7-dehydrocholesterol in corn oil had a manufacturer's assay of 200,000 A.O.A.C. chick units per gm. One gm of this concentrate was diluted with 799 gm of corn oil. This gave sufficient material to carry out both the chick assays and the turkey experiments without making new dilutions. The diluted 7-dehydrocholesterol assayed 250 A.O.A.C. units per gm. The cod liver oil which carried a manufacturer's potency of 250 A.O.A.C. units/gm was assayed by us to contain 255 A.O.A.C. units/gm.

manifests itself at each level of vitamin D intake. In poult nutrition, as in the nutrition of the chick, it would appear that the greater effectiveness of the 7-dehydrocholesterol is demonstrated most markedly when the ration is high in phytin phosphorus and low in inorganic phosphorus.

The efficacy ratios submitted in the last column of table 1 were estimated by fitting a straight line equation,  $Y = 22.173 + 0.013 X$  to the data points of the cod liver oil groups represented in the phytin phosphorus series. The percentage ash values for each level of 7-dehydrocholesterol fed were

TABLE 1

	VIT. D UNITS/ 100 GM DIET	PURE COD LIVER OIL		ACTIVATED 7-DEHYDROCHOLESTEROL		EFFI- CACY RATIO
		Av. wt.	% boneash	Av. wt.	% boneash	
Summary	0	117.4 ( 5) <sup>1</sup>	22.45	117.4 ( 5)	22.45	
expt. 1 with	40	204.3 (17)	33.03	199.5 (14)	33.02	
NaH <sub>2</sub> PO <sub>4</sub> · H <sub>2</sub> O	80	219.3 (12)	40.13	286.4 (19)	43.24	
28 days	160	248.6 (14)	45.29	277.5 (13)	46.52	
	200	275.5 (17)	46.21	295.1 (16)	46.06	
Summary	0	112.4 (20)	23.26	112.4 (20)	23.26	
expt. 2 with	40	127.3 (16)	23.37	128.1 (16)	25.91	6.80
phytin	80	117.5 (15)	24.12	130.8 (19)	27.18	4.65
phosphorus	160	125.2 (14)	24.82	141.3 (17)	27.43	2.40
21 days	200	132.7 (17)	24.61	139.6 (17)	27.70	2.04
	320	140.6 (12)	27.51	148.3 (19)	28.92	1.55
	640	133.3 (18)	27.88	157.3 (19)	31.06	1.04

<sup>1</sup> Figures in parentheses = no. of surviving poults.

plotted on the graph (fig. 1). For any given value, a line is run parallel to the abscissa to a point where it cuts the line of best fit. At this point a line is dropped perpendicular to the abscissa where the corresponding units of vitamin D from cod liver oil necessary to produce an equivalent bone ash may be read. This figure divided by the number of A.O.A.C. chick units of 7-dehydrocholesterol actually fed gives the efficacy ratio.

There are two points of interest to be noted in experiment two. (1) Although the highest level of vitamin D fed in experiment 2 was twice that of the highest level in experiment 1, the

bone ash for this high level (Expt. 2) is less than that for the lowest level of vitamin D in experiment 1. This indicates definitely that calcium magnesium phytate is not a readily available source of phosphorus. One should not, however, infer that the phosphorus of natural plant phytin is necessarily as unavailable (*vide infra*). (2) As the vitamin D level in the diet increases, bone ash likewise increases. However, the increase in per cent bone ash at the higher levels, per unit

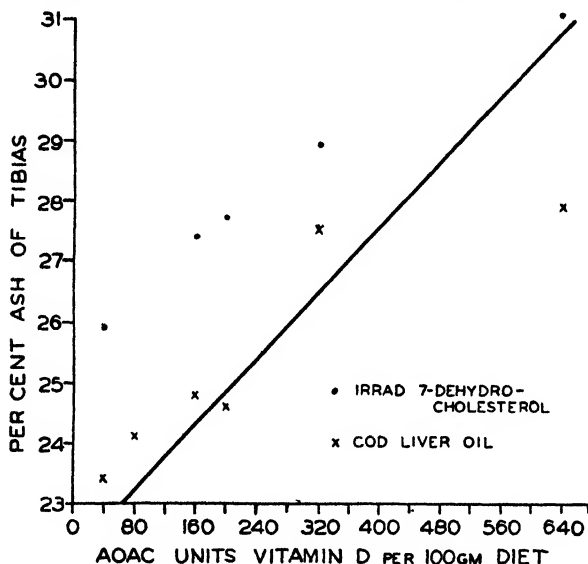


FIG 1 EFFICACY OF 7-DEHYDROCHOLESTEROL AS COMPARED WITH COD LIVER OIL FOR PROMOTING BONE ASH IN POULTS

of vitamin D fed, is considerably less than at the lower levels. Thus we have the greater efficacy ratios for 7-dehydrocholesterol appearing at the lower levels.

Is it possible to correlate these findings with the discrepancies that exist in the literature on the requirements of the turkey poult for vitamin D?

Common ('40) has analyzed some 30 constituents of English poultry feeds for phytin and concludes that in the usual mixed feed some 75% of the phosphorus derived from the cereals

will be in the form of phytic acid. One of us (E.P.S.) has analyzed corn, oats, bran and middlings for phytin with a slightly modified procedure from that used by Common. These analyses seem to run, on the average, some 5% higher than those of Common. This may be due to either a difference in the technique involved or the actual content of phytic acid in the sample analyzed. Common's findings of a considerable spread in the phytic acid content of different samples of feed-stuffs has been confirmed in this laboratory (i.e., different samples of wheat may analyze from 52-72% of the total phosphorus as phosphorus from phytin). That phytin phosphorus from different sources may also vary in its effectiveness in fulfilling the chicks requirements for this mineral is also indicated by some unpublished data of one of us (E.P.S.).

In view of the variations noted above, it is difficult to calculate the absolute values for phytin phosphorus in the various diets used by investigators in studying this problem. However, in the case of Boucher's diet (0.63% total phosphorus) which contained no added mineral phosphorus supplement, there would seem to be little question that the differences noted can be explained on the basis that the poultts were forced to rely on phosphorus from phytin to satisfy their requirements. Bird's diet, on the other hand, contained 1.5% defluorinated superphosphate which, if it were all available, should furnish 0.399% inorganic phosphorus. On the basis of the data presented in table 1, this amount of inorganic phosphorus plus the 0.421% phosphorus (0.201% non-cereal phosphorus) from organic sources in the diet should have resulted in more equal calcification for all sources of vitamin D used. However, Matterson et al. ('45) and Bird et al. ('45) have shown that in the case of the chick and Ellis et al. ('45) in the case of rats, defluorinated superphosphate is not as effective for promoting bone calcification as is tri calcium phosphate or bone meal and if this same condition holds for the poult it would seem reasonable to suppose that the differences noted by Bird can be traced to the differences in

effectiveness of various sources of vitamin D in promoting the utilization of phytin phosphorus in his diet. The diet of Carver and Rhian ('42) was so high in non-cereal phosphorus (0.60%) that one would expect, on the basis of data presented here, to find but slight, if any, difference in the effectiveness of fortified oils as compared with cod liver oil, as indeed they did not. Hammond's ('41) diet (0.48% non-cereal phosphorus) would seem to be approaching the normal requirement of the poult for this type of phosphorus, since his data show a requirement of 80 A.O.A.C. chick units from reference cod liver oil. It should be noted, however, that even at this level of non-cereal phosphorus, activated animal sterols exhibit a greater efficiency, 50 units of activated animal sterol being equivalent to 80 units of reference cod liver oil.

The one discrepancy that stands out in sharp contrast to the hypothesis developed above is the work of Jukes and Sanford ('39). Their diet is calculated to contain 1.0% of total phosphorus and 0.638% of non-cereal phosphorus. This is a greater percentage of non-cereal phosphorus than appears in any of the other diets discussed above and if experimental conditions were comparable should have resulted in a much greater bone ash than these workers report. It seems futile to speculate as to the cause of this disagreement. Nevertheless, it is interesting to note that their later paper (Sanford and Jukes, '44), seems to give us a clue that some factor was operating to depress bone calcification. In their original work, U.S.P. cod liver oil No. 1 when fed at the 80 unit level gave a bone ash of 36.64%. In their later work, with a diet of the same constituent and percentage composition and using U.S.P. Reference Oil No. 2, they report a bone ash figure of 43.4% at the 80 unit level.

One may also note that with their diet No. 2, which more nearly approaches the other diets in mineral content and yet should furnish a sufficient quantity of total phosphorus for bone calcification, they report a lower bone ash at the 80 unit level with Reference Oil No. 2 than they do for diet No. 1 with the same reference oil (40.4% as compared to 43.4%).

## CONCLUSIONS

1. When the diet contains adequate amounts of readily available phosphorus, vitamin D from activated 7-dehydrocholesterol and from cod liver oil produce approximately equal calcification in poults.

2. Vitamin D from activated 7-dehydrocholesterol is more effective than that from cod liver oil in promoting calcification in poults which are forced to rely upon phytin phosphorus to meet their requirements for this mineral. This difference in efficacy ranges from 6.8:1 to 1.04:1 and varies inversely with the level of vitamin D in the feed.

3. With but one exception (Jukes and Sanford, '39) the discrepancies which appear in the literature as to the vitamin D requirement of turkey poults can be explained for the most part at least, on the basis of the availability of the phosphorus used with the various sources of vitamin D in the diet. When the diet is relatively high in the percentage of non-cereal phosphorus, both forms of vitamin D are effective at the lower levels reported. When the diet is relatively high in cereal phosphorus the vitamin D from cod liver oil is less effective and the greater efficacy of activated animal sterols manifests itself.

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# SOME INTERRELATIONSHIPS OF DIETARY IRON, COPPER AND COBALT IN METABOLISM<sup>1</sup>

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The curative effect of cobalt in coast disease in South Australia, enzootic marasmus of Western Australia, bush sickness of New Zealand, nakuriutus of Kenya, pinning disease of Scotland, wasting disease of Western Canada, salt sickness of Florida, lake shore disease of Michigan and Wisconsin and Burton-ail of New Hampshire and Massachusetts (Marston, Thomas, Murname, Lines, McDonald, Moore and Bull, '38; Bowstead, Sackville and Sinclair, '42; Killham, '41; and Keener, Percival and Morrow, '44) has led to the hypothesis that cobalt is an essential element for certain animals. In order to determine the rôle played by cobalt in normal metabolism and its relationship, if any, to iron and copper, this project was undertaken. A microchemical method developed for cobalt analyses on high ash samples made this work possible.

The initial purpose was to estimate cobalt retention at levels in the food characteristic of our diet; to determine the effect of iron and copper on cobalt, and of cobalt on iron and copper retention; and to investigate the disputed interrelationships of iron and copper (Schultz, '40), as well as the validity of application of data on iron and copper anemic animals to normal animals.

<sup>1</sup> The material in this paper was presented before the meetings of the Biological Chemistry section of the American Chemical Society at New York, September 14, 1944.



The plan of the experimentation was to vary systematically the iron, copper and cobalt content of an otherwise good diet and determine the response in food intake, growth, hemoglobin level and metal storage and retention in the healthy albino rat becoming anemic.

## EXPERIMENTAL

### *Test diets*

A basal diet low in iron, copper and cobalt but adequate for fair growth and hemoglobin level was prepared. Because of reports by Ahmad and McCollum ('39), Morris ('40) and Sylvester and Lampitt ('40) of the low cobalt content of milk and milk powder, recently verified by Ellis,<sup>2</sup> the latter was selected as the main constituent of the basal diet.

The composition of the basal diet prepared was as follows: Whole cow's milk powder, 48.5%; washed sucrose,<sup>3</sup> 48.5%; washed Celluration,<sup>4</sup> 2.0%; C.P. sodium chloride, 1.0%; and thiamine hydrochloride, 0.00034%. The washed sucrose was prepared by slowly extracting super-fine sucrose in a long glass Tswett column with 95% ethanol, to which a trace of ammonia had been added, then with ether and finally drying on exposure of a thin layer, protected from dust, to the air. The washed Celluration was prepared by soaking Celluration in approximately 0.2 molar hydrochloric acid solution for 24 hours, with occasional shaking. The solution was filtered off and the extraction repeated the following day. The Celluration then was rinsed several times with redistilled water and dried at 100°C. in Pyrex dishes. The basal metal-low diet was calculated to contain 13% protein, 0.46% calcium, Ca/P = 1.3, 0.0005% thiamine, 0.0008% riboflavin, 8.0 I.U. vitamin A and 4.4 Cal. per gm diet.

<sup>2</sup> Private communications with G. H. Ellis, U. S. Plant, Soil and Nutrition Laboratory, Ithaca, N. Y.; also published in part in Ind. Eng. Chem., Anal. Ed., vol. 17, p. 254 (1945).

<sup>3</sup> Super-fine sucrose, National Sugar Refining Company, New York.

<sup>4</sup> Celluration (70% alpha cellulose, approximately 29% simple and hydro-celluloses and a total ash less than 1.0%), Fischer Scientific Company, N. Y.

This basal diet was analyzed for its content of iron, copper and cobalt and five diets of varying iron, copper and cobalt content devised for the metal studies as shown in table 1. For comparison, the calculated metal content of diet 13 of this laboratory also is given in this table.

### *Animal technique*

Young healthy albino rats of a strain which had been for several generations on adequate diets of whole wheat, whole milk and table salt (diet 13, diet 130 and diet 140<sup>5</sup>), were selected from our stock colony. They were weaned at 28 days of age and killed or placed on the experimental diets at 31 to

TABLE 1

*Metal composition of diets fed animals.*

DIET NO.	METAL CONTENT OF DIETS		
	Iron	Copper	Cobalt
	<i>p.p.m.</i>	<i>p.p.m.</i>	<i>p.p.m.</i>
I (Basal; low Fe + Cu + Co)	2.4	0.67	0.003
II (Basal + Fe + Cu)	4.2	5.5	0.003
III (Basal + Fe + Co)	4.2	0.67	0.083
IV (Basal + Fe + Cu + Co)	3.4	5.5	0.083
V (Basal + Fe + Cu + Co)	4.2	5.5	0.083
13 <sup>1</sup> (Sherman stock diet)	31	6.2	0.08

<sup>1</sup> Calculated cobalt content of diet based on data of Ahmad and McCollum ('39).

33 days old. The animals were divided into six groups so as to equalize, as experimentally practicable, their weight, sex, hemoglobin level and heredity. Animals to be subjected to the experimental diets were placed in individual galvanized wire cages which had been coated recently with aluminum paint. The cages had raised screen bottoms.

Food and distilled water were available to all animals *ad libitum*. Food records were kept. All animals were weighed weekly and their hemoglobin level determined semimonthly.

<sup>5</sup> 31.6 to 32.9% whole cow's milk powder, 63.3 to 65.8% whole wheat and 5.1 to 1.3% sodium chloride.

At 0, 30 and 60 experimental days, an equal number of animals from each of the five diets was killed and the total carcass, minus the gastrointestinal tract, analyzed for iron, copper and cobalt.

### *Analytical methods*

Hemoglobin measurements were made by a modified Hill and Pincock ('41) method, using 0.04% ammonia solution. Spectrophotometric measurements on hemoglobin solutions were made at 540 m $\mu$ .

Diets and rat carcasses were analyzed, without grinding, by a modified Marston and Dewey ('40) method. A sample was wet and dry ashed repeatedly at low temperatures for short intervals of time. The organic-free ash remaining was refluxed with 8 N hydrochloric acid and the iron present extracted with isopropyl ether. Final determination of iron was made with o-phenanthroline. After evaporation almost to dryness of the ether extracted aqueous layer, containing the copper and cobalt, the residue was dissolved in water and the solution formed adjusted to pH 2.5-2.8. The copper was then extracted with dithizone in chloroform and finally determined with sodium diethyldithiocarbamate; at this point, cobalt was extracted with  $\alpha$ -nitroso- $\beta$ -naphthol in carbon tetrachloride. Its final determinization was made with nitroso-R-salt.

### RESULTS AND DISCUSSION

The effect of varying ratios of iron, copper and cobalt in five different diets is shown on food intake and growth (table 2), hemoglobin level (table 3), metal content of fresh carcass after 30 days and 60 days on the experimental diets (table 4) and metal retention (table 5).

Food intake, growth and hemoglobin level decreased almost as rapidly in animals on low copper diet III as in animals on low iron, copper and cobalt diet I. Doubling the iron and increasing the cobalt thirtyfold in the basal diet, in the absence of copper, did not benefit the animal markedly in these respects. The rapid drop of hemoglobin level of animals on

TABLE 2  
*Growth and food intake of animals on diets of varying metal content.*

DIET	NO. OF RATS	Period (days) 0-30 30-60	SEX	AVERAGE BODY WEIGHT PER RAT					AVERAGE DAILY FOOD INTAKE PER RAT				
				0		14		Days rat on diet 30 42		Period (days) rat on diet 0-14 14-30		42-60	
				gm	gm	gm	gm	gm	gm	gm	gm	gm	gm
I	4	2	M	53 ± 3	79 ± 5	108 ± 9	133 ± 6	141 ± 12	83 ± 3	110 ± 6	95 ± 5	127 ± 8	
	4	2	F	52 ± 2	81 ± 5	105 ± 5	123 ± 3	124 ± 11	89 ± 6	112 ± 5	81 ± 10	111 ± 12	
	-	8	M + F	53 ± 3	80 ± 5	107 ± 7	127 ± 5	131 ± 12	86 ± 5	111 ± 6	88 ± 8	119 ± 10	
	4	2	M	52 ± 3	82 ± 4	119 ± 5	153 ± 3	177 ± 6	83 ± 9	126 ± 6	116 ± 2	178 ± 3	
II	4	2	F	50 ± 5	80 ± 2	116 ± 5	139 ± 14	159 ± 19	91 ± 4	131 ± 7	108 ± 11	175 ± 10	
	-	8	M + F	51 ± 4	81 ± 3	118 ± 5	146 ± 9	168 ± 13	87 ± 7	129 ± 7	112 ± 7	177 ± 7	
	4	2	M	52 ± 4	77 ± 4	101 ± 6	118 ± 14	126 ± 19	82 ± 5	105 ± 11	94 ± 10	122 ± 14	
	4	2	F	49 ± 5	73 ± 7	95 ± 11	106 ± 14	102 ± 7	81 ± 10	104 ± 14	77 ± 7	103 ± 0	
III	-	8	M + F	51 ± 5	75 ± 6	98 ± 9	112 ± 14	114 ± 13	82 ± 8	105 ± 13	86 ± 9	113 ± 7	
	4	2	M	55 ± 3	81 ± 8	117 ± 10	149 ± 4	177 ± 3	85 ± 9	126 ± 10	113 ± 3	179 ± 7	
	4	2	F	49 ± 5	80 ± 6	114 ± 8	142 ± 8	160 ± 11	87 ± 7	124 ± 8	107 ± 4	170 ± 1	
	-	8	M + F	52 ± 4	81 ± 7	116 ± 9	146 ± 6	169 ± 7	86 ± 8	125 ± 9	110 ± 4	175 ± 4	
IV	4	2	M	50 ± 4	78 ± 4	112 ± 6	137 ± 5	159 ± 6	88 ± 4	125 ± 4	102 ± 2	165 ± 4	
	4	2	F	51 ± 5	80 ± 10	110 ± 8	127 ± 10	142 ± 13	91 ± 7	128 ± 5	99 ± 5	157 ± 11	
	-	8	M + F	51 ± 5	79 ± 7	111 ± 7	132 ± 8	151 ± 10	90 ± 6	127 ± 5	101 ± 4	161 ± 8	
	4	2	M										

<sup>1</sup> Average deviation of a single observation from the arithmetical mean.

TABLE 3  
Hemoglobin level of animals on diets of varying metal content.

DIET	NO OF RATS		SEX	AVERAGE HEMOGLOBIN LEVEL PER RAT				
	0-30	Period (days) 30-60		0	14	Days rat on diet 30	42	60
I	4	2	M	gm/100 ml 12.65 ± 0.48 <sup>1</sup>	gm/100 ml 9.68 ± 0.52	gm/100 ml 5.66 ± 0.63	gm/100 ml 3.49 ± 0.05	gm/100 ml 2.47 ± 0.14
	4	2	F	12.54 ± 0.48	10.35 ± 0.77	5.73 ± 0.84	4.22 ± 0.45	2.76 ± 0.35
	-	-	M + F	12.60 ± 0.48	10.02 ± 0.65	5.70 ± 0.74	3.86 ± 0.25	2.62 ± 0.25
	4	2	M	12.35 ± 0.68	10.56 ± 0.73	8.59 ± 0.62	8.16 ± 0.04	9.26 ± 0.44
II	4	2	F	12.62 ± 0.15	10.94 ± 0.28	9.32 ± 0.81	9.22 ± 0.44	10.59 ± 0.63
	-	-	M + F	12.49 ± 0.42	10.75 ± 0.51	8.96 ± 0.72	8.69 ± 0.24	9.93 ± 0.54
	4	2	M	12.44 ± 0.85	9.96 ± 1.02	6.19 ± 1.58	5.70 ± 0.05	3.30 ± 0.25
	4	2	F	13.23 ± 0.57	10.52 ± 0.44	6.87 ± 0.92	5.88 ± 0.27	2.96 ± 0.15
III	-	-	M + F	12.84 ± 0.71	10.24 ± 0.73	6.53 ± 1.25	5.79 ± 0.16	3.13 ± 0.20
	4	2	M	12.06 ± 0.52	11.52 ± 0.26	9.02 ± 0.32	7.88 ± 0.16	7.96 ± 0.33
	4	2	F	12.28 ± 0.46	9.62 ± 0.44	7.84 ± 1.20	7.17 ± 1.08	8.11 ± 0.53
	-	-	M + F	12.17 ± 0.49	10.57 ± 0.35	8.43 ± 0.76	7.53 ± 0.62	8.08 ± 0.43
IV	4	2	M	12.24 ± 0.24	11.29 ± 0.38	9.14 ± 0.73	8.79 ± 1.20	9.42 ± 0.83
	4	2	F	12.92 ± 0.51	10.96 ± 1.12	9.54 ± 0.38	9.98 ± 0.74	10.68 ± 0.63
	-	-	M + F	12.58 ± 0.38	11.13 ± 0.75	9.34 ± 0.56	9.39 ± 0.97	10.05 ± 0.73
	8	4	M + F					

<sup>1</sup> Average deviation of a single observation from the arithmetical mean.

TABLE 4

*Iron, copper and cobalt content of animals on diets of varying metal content.*

DIET	DAYS RATS ON DIET	NO. OF RATS	SEX	METAL CONTENT PER RAT			METAL CONTENT PER 100 GM FRESH RAT CARCASS		
				Iron	Copper	Cobalt	Iron	Copper	Cobalt
				mg	μg	μg	mg	μg	μg
—	0	3	M	1.63 ± 0.18 <sup>1</sup>	65.8 ± 4.8	0.06 ± 0.03	3.48 ± 0.16	141.0 ± 0.0	0.11 ± 0.06
		3	F	1.71 ± 0.10	65.5 ± 6.0	0.10 ± 0.06	3.61 ± 0.03	137.0 ± 4.0	0.22 ± 0.16
		6	M + F	1.67 ± 0.14	65.7 ± 5.4	0.08 ± 0.05	3.55 ± 0.10	139.0 ± 2.0	0.17 ± 0.11
I	30	1	M	1.63	63.9	0.55	2.02	79.2	0.68
		2	F	1.63 ± 0.13	49.7 ± 5.6	0.52 ± 0.12	1.92 ± 0.17	58.5 ± 7.2	0.61 ± 0.13
		3	M + F	1.63	56.8	0.54	1.97	68.9	0.65
II	30	1	M	2.66	114.9	0.53	2.61	113.	0.52
		2	F	2.03 ± 0.08	75.4 ± 5.6	0.77 ± 0.21	2.08 ± 0.10	77.1 ± 5.2	0.79 ± 0.21
		3	M + F	2.35	95.2	0.65	2.35	95.1	0.66
III	30	1	M	2.01	58.4	1.16	2.28	66.2	1.31
		2	F	1.66 ± 0.31	51.7 ± 2.7	1.28 ± 0.05	2.09 ± 0.24	66.2 ± 8.5	1.64 ± 0.19
		3	M + F	1.84	55.1	1.22	2.19	66.2	1.48
IV	30	1	M	2.38	108.2	1.45	2.23	102.	1.36
		2	F	1.91 ± 0.09	86.5 ± 3.5	1.44 ± 0.41	2.07 ± 0.05	93.8 ± 6.2	1.57 ± 0.48
		3	M + F	2.15	97.4	1.45	2.15	97.9	1.47
V	30	1	M	3.1	160.	2.3	2.9	150.	2.2
		2	F	2.36 ± 0.53	100.2 ± 6.0	1.53 ± 0.24	2.41 ± 0.35	104.7 ± 14.9	1.57 ± 0.12
		3	M + F	2.73	130.1	1.92	2.66	127.	1.89
I	60	2	M	1.89 ± 0.14	50.1 ± 3.6	0.33 ± 0.03	1.52 ± 0.03	40.7 ± 5.3	0.26 ± 0.01
		2	F	2.13 ± 0.14	46.5 ± 0.1	0.56 ± 0.08	1.94 ± 0.02	42.5 ± 3.2	0.52 ± 0.11
		4	M + F	2.01 ± 0.14	48.3 ± 1.9	0.45 ± 0.06	1.73 ± 0.03	41.6 ± 4.3	0.39 ± 0.06
II	60	2	M	3.21 ± 0.03	143.4 ± 1.3	0.64 ± 0.12	2.01 ± 0.08	89.8 ± 3.4	0.40 ± 0.06
		2	F	3.23 ± 0.20	127.0 ± 1.2	0.77 ± 0.21	2.27 ± 0.13	89.7 ± 11.3	0.57 ± 0.22
		4	M + F	3.22 ± 0.12	135.2 ± 1.3	0.71 ± 0.17	2.14 ± 0.11	89.8 ± 7.4	0.49 ± 0.14
III	60	2	M	2.10 ± 0.32	54.5 ± 6.5	1.17 ± 0.41	1.92 ± 0.02	49.9 ± 2.1	1.03 ± 0.21
		2	F	2.23 ± 0.27	44.8 ± 1.3	1.11 ± 0.10	2.45 ± 0.08	50.0 ± 5.9	1.24 ± 0.22
		4	M + F	2.17 ± 0.30	49.7 ± 3.9	1.14 ± 0.26	2.19 ± 0.05	50.0 ± 4.0	1.14 ± 0.22
IV	60	2	M	2.87 ± 0.01	142.1 ± 7.6	1.80 ± 0.70	1.80 ± 0.01	88.9 ± 4.5	1.12 ± 0.44
		2	F	2.84 ± 0.17	145.7 ± 30.2	2.26 ± 0.24	2.00 ± 0.02	102.0 ± 16.2	1.61 ± 0.25
		4	M + F	2.86 ± 0.09	143.9 ± 18.9	2.03 ± 0.47	1.90 ± 0.02	95.5 ± 10.4	1.37 ± 0.35
V	60	2	M	3.05 ± 0.05	114.4 ± 5.6	1.60 ± 0.70	2.07 ± 0.17	77.6 ± 8.9	1.10 ± 0.5
		2	F	2.90 ± 0.00	126.3 ± 18.0	1.30 ± 0.20	2.32 ± 0.21	102.4 ± 23.6	1.10 ± 0.3
		4	M + F	2.98 ± 0.03	120.4 ± 11.8	1.50 ± 0.50	2.20 ± 0.19	90.0 ± 16.3	1.10 ± 0.4

<sup>1</sup> Average deviation of a single observation from the arithmetical mean.

diet III at the end of 15 days showed inadequate copper storage in the weaned animal. The increase of iron and cobalt of diet III over diet I, while reflected in increased iron and cobalt carcass values, did not change markedly the copper content per 100 gm fresh carcass or copper retention. Cunningham ('31) had found similar results for added inorganic iron alone.

The addition of copper (diet V) to a diet low in copper (diet III) resulted in the expected good biological response. While this increase in copper only slightly increased the iron and cobalt content per 100 gm fresh carcass and cobalt retention, it doubled the iron retention. This observation, on animals becoming anemic, confirms the findings of Bing, Saurwein and Myers ('34), who had obtained results seemingly contradictory to those of Josephs ('32) on anemic animals.

Animals on low cobalt diet II showed a food intake, growth and condition comparable to animals on high cobalt diet V. The addition of cobalt to the diet did not affect the iron and copper content per 100 gm carcass or iron and copper retention. Likewise, the addition of iron and copper to diet I to give diet II did not significantly increase the cobalt content per 100 gm carcass or cobalt retention.

TABLE 5

*Calculated retention of dietary iron, copper and cobalt by animals.*

METAL	DAYS ON DIET	NO. OF RATS	AVERAGE RETENTION PER RAT, DIETS				
			I	II	III	IV	V
			%	%	%	%	%
Iron	30	3	— 12.0 ± 13 <sup>1</sup>	60.0 ± 20	8.0 ± 24	54.0 ± 19	97.0 ± 32
	60	4	35.0 ± 6	73.0 ± 2	31.0 ± 8	73.0 ± 5	66.0 ± 3
Copper	30	3	— 8.3 ± 2.9	1.9 ± 0.9	— 9.8 ± 1.7	2.5 ± 0.5	4.4 ± 1.3
	60	4	— 6.4 ± 0.8	2.5 ± 0.2	— 6.2 ± 1.2	3.0 ± 0.3	2.1 ± 0.3
Cobalt	30	3	75.0 ± 10	90.0 ± 15	7.7 ± 0.6	7.9 ± 0.9	8.9 ± 1.0
	60	4	20.0 ± 5	42.0 ± 6	3.3 ± 0.4	4.9 ± 0.5	3.6 ± 0.6

<sup>1</sup> Average deviation of a single observation from the arithmetical mean.

Food intake and growth were as good for animals on diet IV as on diet V. The slightly lower iron content of diet IV in comparison to diet V was reflected in a lower iron carcass value as well as a lower hemoglobin level in the animal. This small decrease in iron of the diet resulted in no significant change in copper or cobalt content per 100 gm fresh carcass or copper and cobalt retention.

All animals on the five diets increased in total iron and cobalt. However, animals on copper-low diets I and III utilized the copper so poorly that they lost more body copper than they retained from the diets. Animals on the high copper diets II, IV and V stored copper. Likewise, animals on diets I and III had an average copper retention of —6.2 to —6.4%, while those on diets II, IV and V had a 2.1 to 3.0% copper retention. While iron may be rather tenaciously retained by the body, this is not the case for copper which can be lost from body stores in the healthy rat. Although anemic animals generally are considered to retain more metal than normal animals on the same diet, a weaned animal becoming anemic on a copper-low diet actually retained less copper than a litter mate on a good copper diet.

Animals on low copper diets I and III retained only 31 to 35% iron, while animals on high copper diets II, IV and V retained 66 to 73% iron. It is evident that the relatively poor utilization of the iron in the milk diets was due to its low content of copper.

In the case of cobalt, cobalt retention on low cobalt diets (0.003 part per million cobalt) was found to be 30 to 42%, while retention on high cobalt diets (0.08 p.p.m. cobalt) was 3.3 to 4.9%. No retention data for low cobalt diets have yet appeared in the literature. However, Copp and Greenberg ('41) reported that a 12-week-old rat retained, after 96 hours, 2.7% of a single 10  $\mu$ g dose of radioactive cobalt given with a stomach tube.

It is seen that the cobalt content per gm fresh carcass varies with diet, age and sex of the animal. The cobalt content on the fresh basis for 31- to 33-, 60- and 90-day old rats on low



cobalt diet II was found to be 0.002 p.p.m., 0.007 p.p.m. and 0.005 p.p.m., respectively. On high cobalt diet IV, the 90-day-old rat contained 0.014 p.p.m. In table 4, considering all cases, the female rat had a significantly higher cobalt content per gm fresh carcass than her brother. Underwood and Elvehjem ('38) were unable to demonstrate any difference in cobalt content of rats fed mineralized cobalt-low milk and a stock diet. Cobalt storage on excessive cobalt dosing had already been shown (Josland and McNaught, '38).

It had been known that the cobalt requirement of the rat, if any, must be very small. Underwood and Elvehjem ('38) and Underwood ('40) came to the conclusion that the cobalt requirement of the rat was less than 0.6  $\mu\text{g}$  and 0.4  $\mu\text{g}$  per rat per day, respectively. From the data obtained from the present research, the cobalt requirement of the albino rat has been calculated to be less than 0.03  $\mu\text{g}$  per rat per day. This would amount to less than 0.7  $\mu\text{g}$  of cobalt per 1000 Cal.

#### SUMMARY

Five diets of varying ratios of iron, copper and cobalt were fed to young healthy albino rats and the effect of the three metals on food intake, growth, hemoglobin level and metal storage and retention determined. The results were as follows:

1. Low levels of iron or copper decreased food intake, growth and hemoglobin level, but 0.003 p.p.m. to 0.08 p.p.m. of cobalt in the diet showed no difference in these responses.
2. Tentatively, cobalt retention, at levels characteristic of the food supply, was calculated. Cobalt retention on low cobalt diets (0.003 p.p.m. cobalt) was 30 to 42%, while retention on high cobalt diets (0.08 p.p.m. cobalt) was 3.3 to 4.9%.
3. The cobalt content per gm of fresh rat carcass varied with the diet, age and sex of the animals.
4. Iron and copper did not affect cobalt retention nor cobalt markedly iron and copper retention.
5. The iron and copper in the basal milk diet were relatively poorly utilized by the rat. Added copper doubled this

iron retention, while added iron had only a small or insignificant effect on this copper retention, within the range of iron used in these experiments.

6. The body conserved its iron with relatively greater efficiency than its copper.

7. While anemic animals retain more metal than normal animals on the same diet, a normal animal becoming anemic on a copper-low diet retains less copper than a litter mate in better condition on a good copper diet.

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# FACTORS AFFECTING THE OCCURRENCE OF HEMORRHAGIC KIDNEYS DUE TO CHOLINE DEFICIENCY

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The hepato-renal syndrome of choline deficiency is now a well recognized entity in the albino rat. While fatty infiltration of the liver with its sequelae of necrosis and cirrhosis may be produced in rats of any age, renal lesions have only been observed consistently when weanling rats were offered diets which were choline free and of low methionine content but adequate in all other respects. Cholesterol, cystine and a high fat intake increase both the incidence and severity of the disease. This situation has been amply reviewed by Griffith ('41). There are also a few references to such lesions in older animals but these have not been consistent. From the work of Griffith it has seemed that such lesions are most likely to occur at that time when the rate of growth of the animal is at a maximum and when the phospholipid turnover in the kidney is greatest. These concepts have been substantiated by the work of Engel and Salmon ('41) and of Patterson, Keevil and McHenry ('44). The present paper records some observations concerning the effects of other dietary factors and lipotropic substances on the production of such lesions in young rats and the occurrence of these lesions in older rats.

## EXPERIMENTAL

All the rats used in these studies were males of the Vanderbilt strain (Wolfe et al., '38). After weaning they were grown to a weight of 50 gm on a commercial stock chow and then transferred to the experimental diets. They were housed either in separate cages or in "group" cages, 4 rats per cage. Unless otherwise stated, all rats which did not die earlier were killed by decapitation on the eighth experimental day. Liver fat determinations were performed in the manner previously reported (Handler and Dann, '42). A few kidneys from each group were bisected and fixed in formalin, then sectioned and stained with hematoxylin and eosin. Complete histopathological descriptions will not be presented since the lesions, when found, so closely resembled those described by Christensen ('42).

Food consumption was measured daily. However, the figures in the table were calculated only for the first 5 days on the experimental diet since many animals, on becoming ill on the sixth day or later completely stopped eating and comparison of food intake for the entire experimental period with animals which did not show kidney lesions would not be valid. The food intake for rats in group cages was, of necessity, calculated as one-quarter of the food disappearing in each group cage. All values in the tables are expressed in terms of one rat for the 5-day period.

The various diets used are summarized in table 1. The additions noted in table 2 were actually substituted for an equivalent amount of the dietary carbohydrate. In addition all diets except K<sub>10</sub> contained, in mg per kilo, thiamine hydrochloride 5, riboflavin 10, calcium pantothenate 40 and pyridoxine 3.

The system of grading kidney lesions requires some explanation. One plus (+) indicates lesions in only one kidney, usually only a slight mottling and almost invariably this was in the right kidney. Two plus (++) indicates some gross lesions in both kidneys. Three plus (+++) indicates the full blown disease with two, large, hyperemic, almost spleen

colored kidneys while four plus (+++++) has been used to indicate the existence of hemorrhage extending beyond the kidney and into the dorsal wall of the peritoneum. This system is crude but was quite helpful in comparing the effects of different dietary regimes.

TABLE 1  
*Composition of diets.<sup>1</sup>*

	K <sub>1</sub>	K <sub>2</sub>	K <sub>3</sub>	K <sub>4</sub>	K <sub>5</sub>	K <sub>6</sub>	K <sub>7</sub>	K <sub>8</sub>	K <sub>9</sub>	K <sub>10</sub>	K <sub>11</sub>	K <sub>12</sub>	K <sub>13</sub>
Casein	15	15	15	15	15	10	8	6	4	10	12	10	12
Sucrose	58	—	—	—	—	—	—	—	—	84	71	67	57
Starch	—	—	58	—	—	63	65	67	69	—	—	—	—
Glucose	—	58	—	—	—	—	—	—	—	—	—	—	—
Lactose	—	—	—	58	—	—	—	—	—	—	—	—	—
Galactose	—	—	—	—	58	—	—	—	—	—	—	—	—
Cotton oil	15	15	15	15	15	15	15	15	15	—	—	15	15
Salts <sup>2</sup>	5	5	5	5	5	5	5	5	5	4	4	4	4
Crisco	—	—	—	—	—	—	—	—	—	—	10	—	5
Cod liver oil	5	5	5	5	5	5	4	4	4	—	—	4	5
Cholesterol	1.0	1.0	1.0	1.0	0.5	0.5	0.5	0.5	0.5	—	0.5	—	1.0
Cystine	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	—	0.4	—	0.5
Cellulose	—	—	—	—	—	—	—	—	—	2.0	2.0	—	—
Sulfasuxi- dine	—	—	—	—	—	—	—	—	—	1.0	1.0	—	—

<sup>1</sup> The additions noted in table 2 were substituted for an equivalent amount of dietary carbohydrate. In addition all diets except K<sub>30</sub> contained, in mg/kg, thiamine 5, riboflavin 10, calcium pantothenate 40 and pyridoxine 3.

<sup>2</sup> Hubbell, R. G., L. B. Mendel and A. J. Wakeman, 1937, *J. Nutrition*, vol. 14, p. 273.

### *Production of hemorrhagic kidneys in young rats*

When rats were housed in single cages the diet K<sub>1</sub>, which was patterned after those described by Griffith ('41), proved most suitable for the production of kidney lesions. As shown in table 2, all rats on this basal diet showed some sign of kidney damage. However, when the animals were housed in group cages during the experimental period both the incidence and severity of the disease was markedly less than in the single cages, although liver fat concentrations and food intake were of the same magnitude in both groups. The only

explanation which has suggested itself is that the practice of coprophagy provided the rats in group cages with sufficient choline (or source of labile methyl groups) to protect the kidney against injury but not enough to reduce liver fat concentrations effectively. Evidence suggesting that some synthesis, presumably intra-intestinal, of such compounds does occur has recently been furnished by du Vigneaud et al., ('45).

In an attempt to increase the demand for methionine and therefore choline, nicotinamide was added to the diet at a concentration of 0.5%. The rationale for this procedure has

TABLE 2

*Effects of various dietary factors on incidence of hemorrhagic kidneys when used as supplements to the basal ration K<sub>1</sub>.*

SPECIAL SUPPLEMENT	NO. RATS	FOOD INTAKE	LIVER FAT	HEMORRHAGIC KIDNEYS					TYPE OF CAGE
				4 +	3 +	2 +	+	0	
		gm	%						
	24	40	14.7	1	15	7	1	0	Single
	24	36	16.0	0	0	6	8	10	Group
0.5% Nicotinamide	12	21	18.4	0	4	4	3	1	Group
0.5% Nicotinamide	12	22	20.2	0	2	4	3	3	Single
0.25% Inositol	24	37	12.8	17	6	0	1	0	Single
0.25% Inositol	12	34	11.6	1	7	2	1	1	Group
0.25% Inositol + 0.5% nicotinamide	24	22	13.1	8	7	5	3	1	Group
$\alpha$ -Tocopherol 500 $\mu$ g/day	12	36	15.9	0	7	4	0	1	Single
$\alpha$ -Tocopherol 500 $\mu$ g/day + 0.25% Inositol	12	38	9.7	0	2	3	3	4	Single
1% Lipocaine	24	35	9.1	0	1	7	5	9	Single
2% Meat extract	12	40	6.1	0	0	0	0	12	Single
5% Liver extract	12	39	5.4	0	0	0	0	12	Single
Biotin 20 $\mu$ g/day + folic acid 20 $\mu$ g/day	12	39	17.1	0	6	5	1	0	Single
0.15% Choline	24	41	8.7	0	5	4	8	8	Single
0.25% Choline	24	42	4.8	0	0	3	6	15	Single
0.5% Nicotinamide + 0.35% methionine	12	30	7.7	0	1	2	4	7	Group
0.25% Choline + 0.5% nicotinamide	24	26	11.4	0	7	2	8	8	Group
0.5% Choline + 0.5% nicotinamide	24	29	6.1	0	0	0	2	22	Group
Choline 6 mg/day + 0.25 inositol	12	37	8.7	2	3	4	1	2	Single

been discussed by Handler and Dann ('42). This resulted in a considerably increased incidence of hemorrhagic kidneys in the rats in group cages but actually diminished the number seen in single cages. The latter effect appears to have been due to the diminution of food intake caused by the nicotinamide induced methionine deficiency.

*Effects of various lipotropic and alipotropic factors*

The effects of various materials which have been suggested as having lipotropic or alipotropic activity were then tested. The results are also included in table 2. While somewhat reducing the liver fat concentration, inositol actually appeared to increase the incidence and severity of the renal syndrome. In many inositol fed animals the gross hemorrhage which is primarily intra and subcapsular appeared to spread out so that the entire dorsal wall of the peritoneum appeared hemorrhagic. Such animals also invariably showed hemorrhage in the pericardium, lungs and eyesockets as well. These extensive lesions were seldom seen on diets which did not contain inositol.

Tocopherol deficient chicks exhibit capillary damage which is manifested as a generalized exudative diathesis which is, however, prevented by the administration of inositol (Dam and Glavind, '42). Of itself, tocopherol exerted no activity in the present experiments. Tocopherol plus inositol, however, significantly decreased the incidence of hemorrhagic kidneys below that seen in the control groups, in striking contrast to the action of inositol alone. This combination also exerted significant lipotropic action in the liver. Since inositol has been implicated as an active component of "lipocaic" preparations (Abels et al., '43; Gavin and McHenry, '41 a), it was thought of interest to test its activity against choline deficiency hemorrhagic kidneys. Lipocaic was prepared by the method of Clark et al. ('39) from frozen beef pancreas obtained commercially and was included as 1% of the diet. At this level the preparation proved quite efficacious in preventing hemorrhagic kidneys. Unfortunately, the choline con-



tent of the preparation, determined by colorimetric estimation of the reineckate, permitted a daily intake of about 1.8 mg of choline. This level of choline plus 0.25% inositol had relatively little effect on the incidence of renal lesions. However, it cannot be stated whether the activity of the lipocaic was a direct one on the kidney, a sparing action in the liver or, perhaps, due to more complete liberation and availability of the methionine in the dietary casein (Chaikoff et al., '45). We are now attempting to obtain a choline free preparation of lipocaic to test it in this manner. It should be noted that the lipocaic also effectively prevented the accumulation of liver fat as well.

The fatty livers of depancreatized dogs maintained on a diet of lean beef and sucrose do not occur when the insoluble residue from the preparation of commercial meat juice extract are fed, but are manifested when the meat extract is included in the diet (Ralli and Rubin, '42). It seemed of interest to test the effect of meat juice by the present technique and it was found that when meat extract<sup>1</sup> was included as 2.5% of the diet only normal livers and kidneys were found. However choline analysis revealed that the preparation was providing about 17 mg of choline per day which, even in the presence of the inositol may well have been sufficient to account for the results.

Because of the suggestion that biotin increases the demand for lipotropic factors (Gavin and McHenry, '41 b) the effects of biotin,<sup>2</sup> crystalline folic acid<sup>3</sup> and a liver extract prepared according to the directions of McHenry and Gavin ('40) were tested. Neither biotin nor folic acid nor a combination of the two appeared to have any effect. The liver extract, rather than increasing the demand for lipotropic factors, actually prevented the appearance of hemorrhagic kidneys. This again was undoubtedly due to its choline content as it provided at least 11 mg of choline and an unknown amount of methionine

<sup>1</sup> Valentine's.

<sup>2</sup> Merck.

<sup>3</sup> Lederle.

per day. The actual roles of biotin, folic acid and inositol in the homeostatic control of liver fat concentrations has been discussed elsewhere (Handler, '46).

*Influence of the dietary carbohydrate on the incidence of hemorrhagic kidneys*

Griffith ('41) has stated that starch used in place of sucrose in synthetic diets prevented the appearance of hemorrhagic kidneys although no data were presented illustrating the point. We have tested the effects of substituting starch, glucose, lactose and galactose for the sucrose of diet K<sub>1</sub>. In contrast to Griffith's statement both glucose and starch were found to be effective in such diets. When lactose or galactose were

TABLE 3  
*Effects of various carbohydrates in choline deficiency.*  
*All rats were housed in single cages.*

DIET	CARBO- HYDRATE	NO. RATS	FOOD INTAKE	LIVER FAT	HEMORRHAGIC KIDNEYS				
					4 +	3 +	2 +	+	0
			<i>gm</i>	<i>%</i>					
K <sub>1</sub>	Sucrose	24	40	14.7	1	15	7	1	0
K <sub>2</sub>	Glucose	12	38	13.9	0	7	2	3	0
K <sub>3</sub>	Starch	12	37	15.5	0	6	3	2	1
K <sub>4</sub>	Lactose	12	26	7.4	0	0	0	1	11
K <sub>5</sub>	Galactose	12	24	8.1	0	0	0	0	12

used no kidney lesions were found and further, there was only a relatively small increase in liver fat concentration. The daily food consumption was lower on these two diets, and it seems possible that this may account for these effects. While it is not shown in the data (table 3), those rats which ate most and did gain weight showed the largest liver fat concentrations. No rats on these diets ate or grew as well as those on the other diets. The general nutritional inadequacy of lactose as the sole carbohydrate in the diet of the rat has already been reported by Ershoff and Deuel ('44) who found that young rats die after 2 to 3 weeks on such diets. An attempt to determine the underlying mechanism of this nutritional failure will be described in a subsequent report.

*Delayed appearance of hemorrhagic kidneys*

While attempting to determine the optimal dietary conditions for the production of dietary hepatic cirrhosis, studies were made in which various levels of casein were tried (Handler and Dubin, '46). The diets used are summarized in table 1. At casein levels of 15, 10 and 8% (diets K<sub>1</sub>, <sub>6</sub>, <sub>7</sub>) the incidence of deaths due to kidney damage between the sixth and thirteenth day were 65, 40 and 25% of the total number of animals, respectively. No animals died in this manner after the thirteenth day although they were maintained for 120 days on the same diets. When casein was fed as 6% of the diet, (K<sub>8</sub>) no deaths occurred during the apparently critical period found in the series above. Surprisingly however, 17 out of 24 rats then died between the thirty-fourth and forty-fifth day of the experiment with acutely hemorrhagic kidneys. The survivors were sacrificed after 120 days at which time all but one were found to have the characteristic gross "frosted" kidneys indicative of kidney lesions which had healed and this was confirmed by examination of the sections. A second group of 24 animals were placed upon the same diet and groups of 6 were sacrificed at weekly intervals. No kidney lesions were noted in any but the last group sacrificed (28 days). It should also be added that no lesions were found when 4% casein was fed (K<sub>9</sub>).

A delayed development of renal damage due to choline deficiency has also been noted under other conditions. These have been described elsewhere (Handler, '46) and so merit only brief mention in the present report insofar as is pertinent to the discussion. Male rats were fed diet K<sub>10</sub> which contained 10% casein and no fat or B vitamin supplement for 21 days after they had been grown to a weight of 80 gm on a stock ration. They were then transferred to diet K<sub>11</sub> which contained 12% casein, 10% crisco and adequate B vitamins including biotin and folic acid and sacrificed after 8 days. Nine out of 12 animals showed varying degrees of renal damage and all showed markedly fatty livers. Choline alone

was sufficient to prevent the renal lesions but did not quite produce normal liver fat concentrations. Inositol plus tocopherol also reduced the incidence of the kidney lesions and also considerably diminished the liver fat concentrations. While this appearance of hemorrhagic kidneys is not delayed in the same sense as those in the preceding experiment, it does again demonstrate that there is no critical age for the development of such lesions, but rather only a critical situation which may be produced or occur at any age.

*Hemorrhagic kidneys in unilaterally  
nephrectomized rats*

The renal lesions of choline deficiency have only occasionally been noted to develop in mature rats. To check this, diets K<sub>1</sub> (15% casein) and K<sub>7</sub> (8% casein) were each offered to a group of 12 rats after they had attained a weight of 250 gm on a stock ration. The animals were sacrificed after 100 days. Four of the rats on K<sub>7</sub>, exhibited grossly cirrhotic livers and all livers in both groups were extremely fatty although quantitative estimations were not performed. All the animals on K<sub>1</sub> were found to have normal kidneys and 3 rats on K<sub>7</sub> appeared to show slight renal damage.

Forty adult male rats, all of which weighed more than 250 gm were then fed diet K<sub>12</sub> (10% casein) for 6 weeks to deplete them of any choline or labile methyl reserves. They were then subjected to unilateral nephrectomy under ether anesthesia. In each case, the right kidney was removed as this is normally slightly larger than the left kidney (see table 4). They were then changed to diet K<sub>13</sub> (12% casein, 20% fat) and groups of 10 were sacrificed after 8, 10, 12 and 14 days. The results and comparison with control animals on a stock regime are shown in table 4. It will be seen that hemorrhages were noted in all groups but the critical period appeared to lie between the tenth and fourteenth day after nephrectomy. Actually 4 rats were found dead on the thirteenth day with severe renal lesions and are included in the table with the group sacrificed on the fourteenth day. The

large size of the kidneys noted in these rats was undoubtedly due to a combination of hypertrophy and hyperemia such as that seen in choline deficient weanling rats on such diets.

As a control, 12 rats of the same size maintained on a stock ration were subjected to unilateral nephrectomy in the same fashion. Two weeks later they were placed on diet K<sub>13</sub>. All rats survived for another 6 weeks when they were sacrificed. Moderate lesions were found in only one rat.

TABLE 4  
*Hemorrhagic kidneys in unilaterally nephrectomized rats.*  
*Each value is a mean for a group of 10 rats.*

STATE OF RATS	DIET	TIME	LEFT KIDNEY WEIGHT	RIGHT KIDNEY WEIGHT	HEMORRHAGIC KIDNEYS			
					3 +	2 +	+	0
Normal	Stock	days	gm	gm				
		0	0.61	0.67	0	0	0	10
Right kidney removed	Stock	8	1.04	0.71	0	0	0	10
Right kidney removed	Stock	14	1.17	0.70	0	0	0	10
Right kidney removed	K <sub>11</sub>	8	1.08	0.66	0	0	3	7
Right kidney removed	K <sub>13</sub>	10	1.19	0.72	0	1	4	5
Right kidney removed	K <sub>11</sub>	12	1.38	0.70	0	3	3	4
Right kidney removed	K <sub>11</sub>	14	1.70	0.74	3	3	2	2

#### DISCUSSION

The data presented show that renal lesions due to choline deficiency may occur or be induced at any age and are most likely to occur when the food consumption, growth rate and lipid turnover are maximal. Ordinarily, the lipid turnover in the kidney probably parallels that in the entire animal. During the period of hypertrophy after unilateral nephrectomy in adult animals, however, it appeared that the demand for lipotropic factors was at least as great as in the young growing rat and when these were not provided in the diet the typical picture of choline deficiency renal damage was found in the remaining kidney. Further, renal damage due to choline deficiency appears to be an acute rather than a chronic cumulative process. The study of delayed appearance of such lesions on the 6% casein diet (K<sub>6</sub>) confirmed this concept with regard to the morphological aspect of the kidneys, yet demonstrated

that such damage may occur at any time during the life cycle if proper conditions exist, although it is difficult to state precisely what these conditions are.

Despite its lipotropic action in the liver, inositol did not afford any protection against renal damage, but, if anything, appeared to increase both the severity and incidence of the disease. On the other hand a combination of inositol and tocopherol offered some protection. A lipocaic preparation also appeared to offer a greater degree of protection than was warranted by its choline content. It cannot be said whether this was due to a combination of factors or to an actual chemical entity which has been designated as lipocaic. If the preparation contained an appreciable amount of tocopherol and inositol this might have been responsible for the observed activity. This would parallel the inositol-tocopherol relationship in preventing the capillary damage and exudative diathesis of vitamin E deficiency (Dam and Glavind, '42). Dam ('44) has stated that no tocopherol was found in an alkaline hydrolysate of a lipocaic preparation but it is doubtful that any tocopherol could have remained after such drastic treatment nor would it necessarily have split an inositol-tocopherol ether such as that proposed by Milhorat and Bartels ('45).

#### SUMMARY

Rats housed in group cages failed to develop the renal lesions of choline deficiency on a diet which was quite effective when the rats were in single cages. The addition of nicotinamide to this diet resulted in renal lesions even in the rats housed in group cages. While inositol exerted lipotropic activity in the liver, it appeared to increase slightly the incidence of hemorrhagic kidneys due to choline deficiency. A combination of inositol and tocopherol as well as a lipocaic preparation significantly decreased the incidence of such lesions while biotin and folic acid were without effect. Weanling rats on a diet containing 6% casein did not develop hemorrhagic kidneys until after 35 to 45 days, in contrast to rats receiving diets of higher protein concentration which

develop such lesions in 6 to 10 days. While few adult rats developed renal lesions on choline deficient rations, choline deficient adult rats subjected to unilateral nephrectomy uniformly showed such lesions after 10 to 14 days, the period during which the remaining kidney hypertrophied. When adult nephrectomized rats were placed on a choline deficient regime 2 weeks after the operation, renal damage was observed in only one of 12 animals although they were continued on the diet for 6 weeks.

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# A COMPARISON OF FOUR METHODS FOR STUDYING THE URINARY EXCRETION OF THIAMINE

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Excretion of thiamine has been used in various ways as an indication of the state of nutrition with respect to thiamine. The purpose of the work reported here was to compare the results of four methods for studying urinary excretion of thiamine in subjects on a liberal intake of thiamine. The tests used were: (1) basal 24-hour excretion, (2) percentage of intake excreted, (3) response to a 5-mg oral test dose, and (4) response to a 1-mg intramuscular test dose. The level of intake was that recommended for adults by the Food and Nutrition Board of the National Research Council at the time the study was conducted (National Research Council, '43). Several studies have been reported in which investigators have used one or two of these tests and have correlated their figures with the appearance or disappearance of physical symptoms of thiamine deficiency. Melnick ('44) and Holt ('44) have reviewed most of these investigations. No study has been reported in which these four urinary excretion tests were conducted during the same experiment on normal subjects whose intake of thiamine was controlled and constant.

<sup>1</sup> The data in this paper are taken from the thesis presented by Helen H. Giff to the faculty of the Graduate School of Cornell University in fulfillment of the requirement for the M.S. degree, June, 1944. Further details will be found in the thesis.

## EXPERIMENTAL PROCEDURE

Four subjects were maintained on a controlled diet for a period of 44 days, January 6 through February 18, during which time their urinary excretion of thiamine was measured. The level of thiamine intake from food, approximately 600  $\mu$ g per 1000 cal. as calculated from published analyses,<sup>2</sup> remained constant throughout the study. On the first and last days oral test doses of 5 mg of thiamine were administered with breakfast. On the seventh, thirteenth, and forty-third days intramuscular test doses of 1 mg of thiamine were administered just before breakfast.

TABLE 1

*The sex, age, height, weight, calorie and thiamine intake of the four subjects.*

SUBJECTS	SEX	AGE	HEIGHT	WEIGHT	CALORIES <sup>1</sup>	THIAMINE INTAKE FROM FOOD <sup>1</sup>	AVERAGE TOTAL THIAMINE INTAKE/ DAY FOR 43 DAYS <sup>2</sup>
			<i>inches</i>	<i>lbs.</i>		<i>mg</i>	<i>mg</i>
A.M.	F	23	67½	136	2500	1.5	1.7
D.L.	F	30	66	143	2500	1.5	1.7
H.G.	M	26	71	133	3000	1.8	2.0
E.K.	M	24	69	154	3500	2.1	2.3

<sup>1</sup> Approximate calorie and thiamine content of the diets estimated from published analyses. See footnote <sup>2</sup>.

<sup>2</sup> 5-mg oral test dose given on final day of experiment was not included since it did not affect nutritional status during the experiment.

Data concerning the subjects are given in table 1. Physicians of the Department of Clinical and Preventive Medicine at Cornell University found all subjects in good health, that is, that no condition was present which, so far as is known, influences the metabolism of thiamine.

To insure good stores of thiamine at the beginning of the experiment, subjects were given vitamin B complex capsules

<sup>1</sup> Food tables used were: Chatfield, C. and G. Adams, U. S. Dept. Agric. Circ. 549, 1940; Hewston, E. M., and R. L. Marsh, U. S. Dept. Agric. Misc. Pub. 505, 1942; Munsell, H. E., Milbank Mem. Fund Quart., vol. 21, p. 102, 1943. Values for riboflavin content of the wheat germ were obtained from the manufacturer. Analyses made by Doris N. Vidmar 4 months after the conclusion of the experiment indicated that thiamine values were approximately 500  $\mu$ g per 1000 cal. at that time.

for 6 days preceding the experimental period. Each daily supplement furnished 4.5 mg of thiamine hydrochloride, 6 mg of riboflavin, 0.75 mg of pyridoxine hydrochloride, 30 mg of nicotinamide, and 9 mg of calcium pantothenate. No supplement was given on the day just preceding the experimental period.

Urine was preserved with 15 ml of glacial acetic acid per 24-hour specimen. Determinations were made semi-weekly on 3-day pooled samples by the thiochrome method of Hennessy and Cerecedo ('39) as modified by Hennessy ('41). Recovery values were in the range of 93 to 101%.

The experimental diet was planned so that it would be adaptable to different caloric requirements and would furnish adequate amounts of all nutrients except ascorbic acid.<sup>3</sup> The basal diet furnished approximately 1000 cal., 600  $\mu$ g of thiamine, and 1400  $\mu$ g of riboflavin. It consisted of 200 gm of evaporated milk, 100 gm of canned carrots, 100 gm of ground beef chuck, 100 gm of canned pears, 75 gm of dried prunes, 100 gm of canned green beans, 20 gm of Instant Ralston cereal, 20 gm of roasted peanuts, and one medium egg. No foods were allowed ad libitum but additions to the basal diet were planned in units. Each unit addition contained biscuits, cookies, butter, sugar, and wheat germ in amounts to furnish approximately 500 cal., 300  $\mu$ g of thiamine, and 90  $\mu$ g of riboflavin. Each subject was allowed to decide how many of these unit additions he needed to satisfy appetite and energy requirements, and throughout the experiment kept his food intake constant as nearly as could be determined by calculation. The calorie and thiamine intakes of each subject are shown in table 1. The diets as eaten furnished slightly more than 1000  $\mu$ g of thiamine per 1000 non-fat cal. Approximately 40% of the calories were supplied by fat.

<sup>3</sup> Vitamin C metabolism studies which were being conducted concurrently on the same subjects necessitated a diet low in ascorbic acid, but daily supplements of synthetic ascorbic acid made the level of intake 53 mg. The subjects were saturated with ascorbic acid during the pre-experimental period.

to be similar, while D.L. consistently excreted less than the others. The lowest value for D.L., 100  $\mu\text{g}$  per 24 hours on a 3-day pooled sample, approached the "critical level", 90  $\mu\text{g}$  per 24 hours, which is the lowest figure considered by Mason and Williams ('42) to indicate adequate nutrition. All other values were well above this "critical value".

TABLE 3  
*Summary of results.*

URINARY EXCRETION TEST	SUBJECTS			
	A M.	D.L.	H G.	E.K.
Basal 24-hr. excretion <sup>1</sup> ( $\mu\text{g/day}$ )				
Range	254-162	184-100	270-153	282-188
Average	197	135	195	210
% of intake excreted <sup>1</sup>				
Range	17-11	12-7	13-9	15-9
Average	13	9	11	10
Response to 5-mg oral test dose (% recovery) <sup>2</sup>				
1st day	29	33	37	40
44th day	16	16	15	22
Response to 1-mg intra- muscular test dose % recovery <sup>2</sup>				
6th day	12	30	20	28
13th day	19	10	22	20
43rd day	14	8	13	21
$\mu\text{g/day}$				
6th day	518	540	544	657
13th day	494	290	468	528
43rd day	328	193	281	403

<sup>1</sup> For final 4 weeks of experiment.

<sup>2</sup> Percentage of test dose excreted in 24 hours following administration.

<sup>3</sup> Extra excretion obtained by subtracting basal excretion from the value for the 24-hour collection period, and then expressed as percentage recovery of the test dose. Basal excretion was interpolated from the values for the preceding and succeeding analyses.

*Percentage of intake excreted*

Percentage of the intake excreted by the four subjects of this study is shown in table 3. The range of values for male subjects was 9 to 15%. Melnick et al. ('39) noted values of 15 to 27% for normal men, and Jolliffe et al. ('39) observed that five men excreted 13.1 to 25.7% of their intake on a diet estimated to contain 564  $\mu$ g of thiamine per 1000 cal. The range for the two female subjects of this study was 7 to 17% as compared with values of 9 to 31% observed by Melnick et al. for normal women.

*Response to 5-mg oral test dose*

Responses to the 5-mg oral test doses administered on the first and last day of the study are shown in table 3. Recovery was calculated by the method most commonly used, that is, the total thiamine excreted in 24 hours following administration of the dose was used to calculate the percentage recovery of the dose. The larger excretions on the first day were undoubtedly caused by vitamin supplements given the subjects during the week preceding the experimental period. Values for the last day ranged from 15 to 22% of the test dose. Melnick et al. ('39) found that 15 normal men averaged 14% and 10 normal women 12% excretion of a similar test dose. In another study Melnick and Field ('42) observed that 23 normal adults with good stores excreted 7 to 30% and 14 normal adults with poor stores excreted 1 to 7%. Six other normal adults studied by Melnick ('42) excreted 13 to 20.8% of the dose. Values for excretion of a 5-mg oral test dose by subjects at the end of this study, when compared with the aforementioned figures could be interpreted to mean that thiamine reserves were adequate.

*Response to 1-mg intramuscular test dose*

One-mg intramuscular test doses were administered on the seventh, thirteenth and forty-third days of the period. Responses to these doses are shown in table 3, both in milli-

grams of thiamine excreted in 24 hours, and as extra excretion due to the dose. Extra excretion, obtained by subtracting basal excretion from the value for the 24-hour collection period, was expressed as percentage recovery of the test dose. Basal excretion was obtained by interpolation.

Percentage recovery of a 1-mg intramuscular test dose by the subjects of this study is shown in table 3. These estimates are probably less accurate for the first two than for the last of the test doses, since the basal excretion fell rapidly during the first 2 weeks. Under these conditions, the figure for basal excretion on the day of the test dose, which must be interpolated to calculate percentage recovery, can be only a fair guess of what the excretion actually was. Therefore an average of two values would probably give a more suitable figure for comparative purposes than either value alone. These averages for the four subjects are: 15% for A.M., 20% for D.L., 21% for H.G., and 24% for E.K. In all cases these figures are higher than recoveries observed when a third intramuscular test dose was administered at the end of the experiment. This lower recovery is particularly notable in the cases of D.L. and H.G. The recovery value of 8% for D.L. is in the range of values observed for persons with such depleted stores that symptoms of thiamine deficiency were present (Mason and Williams, '42). The response of H.G., 13%, might indicate that some depletion had occurred, particularly since he responded to the first two test doses with much higher recoveries. Subject A.M. responded in substantially the same degree to all three of the tests. Her recovery value of 14% of the last dose might be considered a borderline figure, but probably indicated sufficient stores when interpreted in the light of her comparatively low responses to the first two doses when her reserves must have been adequate. The response of E.K. to the last test dose, 21%, was within the range of 15 to 45% recovery observed by Mason and Williams ('42) for subjects on unrestricted diets with large thiamine supplements.

Average responses to the first two test doses ranged from 415 to 592  $\mu\text{g}$  of thiamine in 24 hours. These figures are comparable to those observed by Williams et al. ('42) at a similar level of intake. Excretion values for the third test dose administered 30 days after the second and at the end of the study are more comparable to those observed by Williams et al. on an intake of 400  $\mu\text{g}$  per 1000 cal. The low response of D.L., 193  $\mu\text{g}$ , is even less than the reported values on an intake of 300  $\mu\text{g}$  per 1000 cal.

#### *Comparison of results with four procedures*

Four tests were used in this study to determine the state of the thiamine reserves, and the results of the tests were interpreted in the light of the rather meager data in the literature. On this basis, the four tests were not in agreement concerning the status of the subjects at the end of the study. Basal 24-hour excretion and responses to the 5-mg oral test dose indicated that for all subjects reserves of thiamine were adequate. This is what one would expect if the Food and Nutrition Board was justified in considering this level of intake more liberal than necessary, as is indicated by the reduction in recommended daily allowances for thiamine (National Research Council, '45). Calculation of the percentage of intake excreted suggested that subject A.M. had normal reserves but that the other three subjects had borderline or sub-normal reserves. Responses to the 1-mg intramuscular test dose suggested that only one subject, E.K., had adequate stores. No subject was judged to have normal reserves of thiamine on the basis of all four criteria.

When results for the four urinary excretion tests are considered independently of the suggested normal values in the literature, the results obtained by different methods agree reasonably well, i.e. each subject behaved fairly consistently. Throughout the study the female subject, D.L., excreted less thiamine than the other subjects and exhibited smaller responses to the test doses. The excretion values of the male subject, E.K., were in general appreciably higher than for



any of the other subjects, and his responses to the test doses given on the last 2 days of the experiment were particularly so. Possibly these subjects differed in their renal thresholds for thiamine, but our data provide no information on this point. Though all subjects maintained the same thiamine-calorie ratio in accordance with the recommended daily allowances then current (National Research Council, '43), the total intake of thiamine and calories was higher for E.K. than for any of the others. On the basis of the present recommended allowances (National Research Council, '45) which do not maintain a constant proportion of thiamine to calories, this subject had a greater margin of safety in his thiamine intake than the other subjects.

The apparent disagreement in results observed when nutritional status was judged by these four criteria suggests that the range of normal values for these tests has not yet been satisfactorily established. The range of individual variation may be so great, however, that results from four subjects are insufficient to warrant definite conclusions.

#### SUMMARY

Four normal adults were maintained for 44 days on a controlled diet which was estimated to furnish 600  $\mu$ g of thiamine per 1000 cal. Basal 24-hour urinary excretions of thiamine were higher at the first of the period than at the end. Supplements of thiamine given during the pre-experimental period undoubtedly caused elevations in values for the first 2 weeks of the study.

Four urinary excretion tests for thiamine were studied. Values for 24-hour excretions of thiamine for the last 4 weeks ranged from 100 to 224  $\mu$ g per day. Average percentages of thiamine intake excreted ranged from 9 to 13%. Responses to a 5-mg. oral test dose at the end of the study ranged from 15 to 22% recovery in 24 hours. Responses to 1-mg intramuscular test doses toward the beginning of the study ranged from 15 to 24% recovery. At the end of the study they ranged from 8 to 21% recovery, or 193 to 403  $\mu$ g excretion in 24 hours.

When the urinary excretion values recorded for "normal" subjects were used as standards for comparison, the nutritional status of these subjects with respect to thiamine was not judged to be the same by all four criteria. More data are needed to establish the range of normal values for these urinary excretion tests, and their relative sensitivity.

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# THE EFFECT OF VARIATIONS IN THE LEVEL OF DIETARY CALCIUM UPON THE GROWTH OF YOUNG RATS RECEIVING ATABRINE

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Several interesting relationships between nutrition and the oral administration of atabrine have been demonstrated. Seudi and co-workers ('44) have shown that doses of atabrine administered by stomach tube were less toxic to animals fed a high-protein, low-fat diet than to animals receiving a low protein or a low-protein, high-fat diet. By incorporating atabrine in the ration, Hegsted, McKibbin and Stare ('44a) demonstrated retarded growth of young rats as well as some degree of liver damage when the level of atabrine in the ration exceeded 25 mg/100 gm ration (about 25 mg/kg body weight in weanling rats). Further studies with young rats showed that the inclusion of a relatively large proportion of atabrine in a ration deficient in choline prevents the development of hemorrhagic kidneys (Hegsted, McKibbin and Stare, '44b) and that atabrine exhibits a thiamine sparing action (Hegsted, McKibbin and Stare, '45).

Presented here are the results of studies in which the calcium content of the diet was modified and the effect upon the growth of young rats with and without additions of atabrine determined.

## EXPERIMENTAL

Weanling male rats of the Sherman strain were housed in individual cages and fed the experimental rations *ad libitum*. Four to eight animals constituted a group. They were weighed twice weekly and during two studies records of food consumption over 4-week periods were made.

The basic purified ration was that described by Hegsted et al. ('44b). The calcium content of this ration (0.55%) was decreased to 0.15% in the low calcium rations by the substitution of 3% of a salt mixture low in calcium for the one previously used and was raised to 1.35% in the high calcium rations by the addition of 2%  $\text{CaCO}_3$  at the expense of sucrose.

An alcohol solution of the water soluble vitamins was dried on the ration as described in the foregoing publications and fat soluble vitamins in corn oil were given by dropper twice weekly.<sup>1</sup> Atabrine hydrochloride dissolved in alcohol was added to the ration at levels of 40 or 60 mg per 100 gm.

Determination of atabrine in liver and spleen was made by extracting a tissue homogenate (McIlvaine's buffer, pH 7.8) with ethylene chloride; atabrine was extracted from this with 0.3 N HCl for reading in the photofluorometer.

The pH of the intestinal content of certain of the experimental animals was determined as follows: the animals were sacrificed individually and the intestinal tract excised and divided into three segments, upper and lower small intestine and cecum. The contents of each of these portions were then washed immediately into a small beaker with 20 ml distilled water and the pH determined by means of a glass electrode. Values found for the lower small intestine and cecum were consistent for animals on a given diet; in the upper portion of the tract the range of variation was somewhat greater and stomach pH figures were discarded because of the extreme variation found, presumably due to differences in time since the ingestion of food.

<sup>1</sup> We are indebted to Merck & Co., Inc., Rahway, New Jersey, for furnishing the crystalline B-vitamins and alpha tocopherol, and to Abbott Laboratories, North Chicago, Illinois, for halibut liver oil.

## RESULTS AND DISCUSSION

As in the experiments cited previously, the addition of 40 to 60 mg of atabrine per 100 gm purified ration resulted in a decrease of 20 to 30% in the weight gain of young rats (table 1). It may be seen that alterations in the level of dietary calcium intake were accompanied by differences in tolerance to atabrine as measured by growth. In the series of four experiments, rats receiving 40 or 60 mg % atabrine with the high calcium diet attained but 51%–63% of the weight gain of their respective controls not receiving atabrine. On the other hand, animals receiving the same amount of atabrine in the ration but maintained at the lower calcium levels grew 67%–77% as well as the appropriate control groups. Statistical treatment of these data by means of the “t test” showed these differences significant with the possible exception of experiment 2.

Food intake records (table 2) show that the rats receiving atabrine consumed somewhat less than the control groups; however the efficiency of food utilization was variable. The actual amount of atabrine ingested by the low and high calcium groups of animals was comparable, about 4 mg per day. Despite the fact that the low and high calcium groups ingested the same amount of atabrine, determinations of atabrine concentration in liver and spleen showed a difference between the two groups. It may be seen from table 3 that rats on the high calcium diet stored on the average twice the amount of atabrine in the liver as did animals at the low calcium level. These differences again were found to be statistically significant. The concentration of atabrine in the spleen reflected the same trend although the contrast between the two groups was less marked.

It is observed that the atabrine concentration in the livers of groups receiving either the high or low calcium ration was no higher after 63 days on the diet than after only half that time. The concentration of atabrine in the liver varied with the individual animal and with the calcium level of the diet. In these experiments it appeared to be attained within a com-

TABLE 1

*Average gain in weight of young rats on various levels of calcium, atabrine, and other supplements.*

EXPERIMENT NO.	RATION			NO. ANIMALS	DURATION OF EXPERIMENT	AVERAGE WEIGHT GAIN	PLUS ATABRINE AS % OF CONTROLS
	Calcium	Atabrine	Supplement				
	%	mg/100 gm ration			days	gm	
1	0.15	..	None	5	63	223	..
	0.15	40	None	4		173	77
	0.55	..	None	4		216	..
	0.55	40	None	5		147	68
	1.35	..	None	4		247	..
	1.35	40	None	5		125	51
2	0.15	..	None	6	51	155	..
	0.15	60	None	5		105	67
	0.15	..	6% ammonium salt mixture <sup>1</sup>	5		144	..
	0.15	60	6% ammonium salt mixture	5		85	59
	1.35	..	None	6		164	..
	1.35	60	None	5		95	58
	1.35	..	6% ammonium salt mixture	5		140	..
	1.35	60	6% ammonium salt mixture	5		82	59
3	0.15	..	None	6	46	171	..
	0.15	40	None	6		121	71
	0.15	..	2% citric acid	6		149	..
	0.15	40	2% citric acid	5		104	70
	1.35	..	None	4		162	..
	1.35	40	None	6		102	63
	1.35	..	2% citric acid	5		157	..
	1.35	40	2% citric acid	6		115	73
4	0.15	..	None	7	30	110	..
	0.15	40	None	8		80	73
	1.35	..	None	7		97	..
	1.35	40	None	8		57	58
	0.15	..	1% MgCO <sub>3</sub>	3		82	..
	0.15	40	1% MgCO	3		58	71

<sup>1</sup> Two per cent NaHCO<sub>3</sub> was used instead of the ammonium salts for 30 days; when it was found that there was no effect either upon growth or the intestinal reaction, the mixture of equal parts ammonium carbonate and ammonium chloride was substituted at a 6% level.

paratively short period of time and was not influenced by long-continued ingestion of atabrine. It might be suggested, therefore, that the differences in tissue concentration and growth rate manifested by animals at different calcium levels were due less to changes in absorption of atabrine from the intestinal tract than to some factor influencing the fate of the drug after entering the body.

TABLE 2

*Food consumption and efficiency of food utilization on varying levels of calcium and atabrine.*

RATION	AVERAGE FOOD INTAKE		EFFICIENCY OF FOOD UTILIZATION	
	Experi- ment 1	Experi- ment 4	Experi- ment 1	Experi- ment 4
	gm'/day	gm'/day	gm food/ gm gain	gm food/ gm gain
0.15% calcium	12.1	10.4	2.8	2.8
0.15% calcium + 40 mg % atabrine	10.9	9.5	3.5	3.4
0.55% calcium	13.4	....	3.4	...
0.55% calcium + 40 mg % atabrine	10.5	....	3.3	...
1.35% calcium	15.5	14.0	3.4	4.2
1.35% calcium + 40 mg % atabrine	11.2	8.6	4.0	4.2
0.15% calcium + 1% MgCO <sub>3</sub>	....	6.9	...	2.7
0.15% calcium + 1% MgCO <sub>3</sub> + 40 mg % atabrine	...	5.3	...	2.8

In this connection it should be stated that several of the animals on the high calcium ration without atabrine were found to have suffered renal damage. The kidneys were hypertrophied and numerous stones were observed in the kidneys and in the bladder. It is possible that the rats on the high calcium ration were unable to excrete as much atabrine via this route as was the group on the low calcium diet.

In experiments 2, 3, and 4 an attempt was made to determine whether or not the effect of the calcium content of the diet in establishing the degree of atabrine toxicity might be due to altered pH of the intestinal contents. It is known that a high proportion of calcium in a purified diet promotes a



more alkaline intestinal reaction. Substances thought to produce acidic or basic reaction were added to the low and high calcium rations and pH determinations made on the intestinal contents of animals fed these diets for a week or more. Results in terms of the pH of the intestinal contents are presented in table 4. Of the substances tested, only

TABLE 3

*Concentration of atabrine in liver and spleen of rats receiving 40 mg % atabrine on low and high calcium diets.*

EXPERIMENT NUMBER	NO. ANIMALS	DURATION OF EXPERIMENT	ATABRINE				
			Liver		Spleen		
			0.15% Ca	1.35% Ca	0.15% Ca	1.35% Ca	
		days	$\gamma/gm$	$\gamma/gm$	$\gamma/gm$	$\gamma/gm$	
1	4	63	235	2000	870	1520	
			872	1525	1170	1310	
			520	1300	1040	1180	
			450	1000	730	1180	
			Average.....				
4	8	30	631	1360			
			733	1904			
			593	1070			
			721	1366			
			528	1624			
			1275	1289			
			1147	1520			
			709	1675			
Average.....				792	1476		

MgCO<sub>3</sub> and the CaCO<sub>3</sub> of the high calcium ration produced an alkalinity of the intestine, and that was apparent only in the lower small intestine and cecum. The inclusion of atabrine in a ration did not affect the intestinal pH of the animal.

The growth data for rats receiving citric acid, a mixture of equal parts of ammonium chloride and ammonium carbonate, or magnesium carbonate in addition to the high and low calcium levels with and without atabrine are included in

table 1. It may be seen that the ammonium salts did not affect the response to the high calcium ration but did increase somewhat the growth inhibition due to atabrine on the low calcium ration. The citric acid, conversely, improved the high calcium ration so that rats receiving atabrine plus high calcium with citric acid grew as well as did the low calcium animals receiving atabrine, with or without citric acid. This may indicate that acidity or alkalinity of the diet influences

TABLE 4

*The pH of intestinal contents of rats maintained on varying levels of calcium and with certain salt supplements.*

CALCIUM CONTENT OF RATION	SUPPLEMENTS	NO OF ANIMALS	pH OF INTESTINAL CONTENTS		
			Upper small intestine	Lower small intestine	Cecum
0.15%	None	16	6.34 $\pm$ 1.02	6.64 $\pm$ 0.32	6.73 $\pm$ 0.28
	1% NaHCO <sub>3</sub>	2	6.28	6.63	6.62
	1% Al(OH) <sub>3</sub>	2	5.95	6.87	6.60
	1% MgCO <sub>3</sub>	2	6.24	7.63	8.05
	3% NH <sub>4</sub> Cl	6	6.19	6.79	6.61
	3% (NH <sub>4</sub> ) <sub>2</sub> CO <sub>3</sub>				
	2% citric acid	5	6.45	6.74	6.89
0.55%	None	2	6.54	6.95	7.40
	1% citric acid	2	6.78	7.19	7.32
	2% citric acid	2	6.63	7.04	7.28
1.35%	None	15	6.53	7.58	7.95
	3% NH <sub>4</sub> Cl	2	6.60	8.01	8.19
	3% (NH <sub>4</sub> ) <sub>2</sub> CO <sub>3</sub>				
	2% citric acid	5	6.51	7.89	8.26

atabrine absorption or storage, although citric acid and the ammonium salt mixture could not be shown to influence the pH in the intestinal tract. A small group of animals given the low calcium ration to which 1% MgCO<sub>3</sub> was added (experiment 4) showed no significant growth difference in response to atabrine from the low calcium controls. However, MgCO<sub>3</sub> was shown to produce an intestinal reaction as alkaline as that found on the high calcium diet. It is not clear, therefore,

whether the calcium ion exerts some specific effect upon the fate of ingested atabrine, whether the discrepancies were due to defective excretion, or whether the augmented toxic effect observed on the high calcium diet is in some way associated with the intestinal pH.

A few attempts were made to study *in vitro* the intestinal absorption of atabrine by passing solutions of the drug through an isolated loop of fresh intestinal tissue suspended in a buffer medium. Comparison with xylose diffusion curves conducted simultaneously suggest that, under the conditions imposed, atabrine passed through the membrane by diffusion. It was of interest that characteristic peristaltic movements continued in the isolated intestinal loop for more than an hour after the addition of atabrine. Tissue similarly placed in the buffer but without atabrine did not exhibit this action. This observation is of interest as Keogh and Shaw ('44) have reported that the action of calcium ions in causing the relaxation of smooth muscle is reversed in the presence of atabrine or quinine. Gastrointestinal symptoms following administration of atabrine to man have been reported (Goodman and Gilman, '41).

#### CONCLUSIONS

1. The inhibition of growth of young rats by the addition of 40 or 60 mg % atabrine to a purified ration was significantly greater in animals maintained on a high calcium ration, 1.35%, than when the ration contained a calcium level of 0.55% or 0.15%.

2. The average concentration of atabrine in the liver and spleen of rats on the high calcium diet was considerably greater than that found for animals receiving the low calcium diet, despite a comparable atabrine intake.

3. The concentration of atabrine in the liver in these experiments was as high at 30 days as after 63 days.

4. Animals on the high calcium diet had a more alkaline reaction in the lower small intestine and cecum. However, by the addition of various salts it could not be shown that in-

testinal pH was a major factor in affecting atabrine toxicity. Some animals on the high calcium diet exhibited renal calculi which may have impaired the excretion of atabrine. Hence it is not clear whether the increased toxicity of atabrine on a high calcium intake is due to increased absorption, decreased excretion, or to a specific effect of calcium on atabrine metabolism.

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# PHOSPHORUS METABOLISM OF PRESCHOOL CHILDREN

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This report on phosphorus metabolism is the fourth in a series on the effect of changes in the ascorbic acid and citrate content of the diet on the ascorbic acid, citric acid, calcium, phosphorus, and nitrogen metabolism of eight preschool children. The effect on ascorbic acid, citric acid, and calcium metabolism have been reported by Meyer and Hathaway ('44), Metcalf and Hathaway ('45) and Watson and associates ('45), respectively.

## EXPERIMENTAL

The general plan of the complete experiment has been reported in detail by Meyer ('43), and a summary of the facts pertinent to the calcium and phosphorus metabolism studies given by Watson and associates ('45). Briefly, they are these: eight children between the ages of 38 and 55 months and weighing 29 to 50 pounds were maintained on a basal diet believed to be adequate in all nutrients except ascorbic acid. The first year the children A, B, C, and D were given 800 ml of milk daily; the second year, subjects E, F, G, and H received only 500 ml. Through changes in the basal diet, particularly an increase in the meat and eggs, an attempt was made to maintain the level of phosphorus in the low-milk diet. There was greater variation from subject to subject in the phosphorus intakes on the low-milk diet, however, be-

cause these changes were in the basal diet, and somewhat less of the basal foods was consumed by subject H throughout the study, and by subject G during the first 4 and last 5 weeks. Also, the Ry-Krisp and Toasted Wheat Wafers, allowed ad libitum, contained significant amounts of phosphorus. Supplements of crystalline ascorbic acid (100 mg), potassium citrate (3.38 gm), or both were given as indicated in table 1. During the last 2 weeks, the children of the low-milk group were given orange juice in place of crystalline supplements.

The methods of collecting and preparing samples for analysis have been described by Metcalf and Hathaway ('45) and Watson and associates ('45). With the exception of the milk samples for the second year, all samples for phosphorus analyses were ashed using essentially the method of Gerritz ('35). The phosphorus content of the ashed samples was determined by a modification of the method of Fiske and Subbarow ('25), adapting it to the photoelectric colorimeter. Exact timing for the development of the color in samples and standards was used.

The milk samples for the second year were ashed in a muffle furnace at about 400°C., as suggested by Peters and Van Slyke ('32). The phosphorus values were much lower by this method, and are believed to be unreliable. The milks for the 2 years were specially prepared in the University Dairy Department.<sup>1</sup> They were obtained from the same herd, at the same time of year, and had the same average nitrogen and calcium content. Since the calcium:phosphorus ratio in milk under these conditions should be constant, it seemed advisable to use the average found for phosphorus in milk for the first year in calculating the phosphorus intakes for the second year.

#### RESULTS AND DISCUSSION

The data obtained in this study are presented in table 1. Data for the four preliminary periods have been omitted.

<sup>1</sup> Specially prepared by Prof. E. S. Guthrie to preserve the ascorbic acid content. See Sharp, Hand, and Guthrie ('39).

*Average daily phosphorus balances for eight preschool children.*

SUBJECT A

SUBJECT B

SUBJECT C

SUBJECT D

DIET PERIOD	INTAKE	EXCRETION		INTAKE	EXCRETION		INTAKE	EXCRETION		INTAKE	EXCRETION		RETENTION	
		mg	feces		mg	feces		mg	feces		mg	feces		
I <sup>1</sup>	5	1374	537	1249	750	434	65	1258	670	454	134	777	30	
	6	1321	607	1343	690	404	249	1366	662	447	257	739	415	
	10	1321	605	1306	742	410	154	1321	774	418	129	734	433	
	11	1294	588	1245	702	430	113	1296	797	492	147	723	346	
	12	1208	515	1168	693	403	72	1203	631	406	166	747	368	
Av.		1299	562	1262	715	416	131	1289	693	429	167	748	398	
II <sup>2</sup>	7	1299	578	1266	711	417	138	1299	626	412	261	782	357	
	8	1303	569	1265	698	394	193	1312	753	406	153	748	363	
	9	1309	519	1292	718	414	160	1237	729	395	113	734	359	
	13	1254	605	1212	725	390	97	1266	715	491	60	759	339	
	14	1297	614	1235	725	373	137	1282	732	438	112	756	354	
Av.		1292	577	1258	715	398	145	1257	647	431	179	700	350	
III <sup>3</sup>	15	1270	609	1237	779	546	—86	1257	647	431	179	700	350	
	16	1307	637	1267	719	404	144	1289	748	431	110	787	325	
	17	1306	591	1268	705	408	155	1299	631	505	103	764	400	
	18	1294	612	1257	734	453	70	1282	675	476	131	727	358	
	19	1264	617	1226	708	379	139	1253	738	447	68	720	332	
Av.		1281	622	1248	736	399	113	1262	692	477	93	739	360	
IV <sup>4</sup>	20	1262	592	1227	663	481	83	1255	697	485	73	752	357	
	Av.		1269	610	1234	702	420	112	1257	709	470	78	737	350
	SUBJECT F													
	I <sup>1</sup>	7	1077	580	1077	601	388	88	1077	578	409	90	961	521
		8	1089	576	1085	588	388	109	1085	540	448	97	982	535
9		1114	588	1109	586	440	83	1109	566	427	116	998	548	
Av.			1093	581	1090	592	405	93	1090	561	428	101	980	535
II <sup>2</sup>		5	1120	599	1131	597	409	125	1135	534	494	107	985	513
	6	1080	573	1076	592	415	69	1087	536	490	61	956	524	
	10	1082	601	1078	567	419	92	1071	564	415	92	977	547	
	11	1091	602	1087	576	436	75	1037	540	398	99	967	529	
	12	1099	594	1095	593	458	44	1066	564	419	83	990	548	
Av.		1094	594	1093	585	427	81	1079	548	443	88	975	532	
III <sup>3</sup>	13	1096	610	1092	596	404	92	1039	539	366	134	988	534	
	14			1084	570	352	162	1019	547	363	109	976	513	
	15	1101	600	1093	588	424	81	1019	560	343	107	971	514	
	Av.		1098	605	1090	585	393	112	1023	549	357	117	978	520
	IV <sup>4</sup>	16	1096	593	1091	583	442	66	901	522	339	40	951	534
17		1094	595	1097	567	433	97	896	529	320	47	948	518	
18		1106	580	1100	565	419	116	905	500	322	83	952	505	
Av.			1099	589	1096	572	431	93	901	517	327	57	950	519
V <sup>5</sup>		19	1129	613	1118	605	421	92	928	534	301	93	950	527
	20	1139	635	1127	607	393	127	922	520	314	88	940	554	
	Av.		1134	624	1122	606	407	109	925	527	91	945	540	
	SUBJECT G													
	I <sup>1</sup>	7	1077	580	1077	601	388	88	1077	578	409	90	961	521
8		1089	576	1085	588	388	109	1085	540	448	97	982	535	
9		1114	588	1109	586	440	83	1109	566	427	116	998	548	
Av.			1093	581	1090	592	405	93	1090	561	428	101	980	535
II <sup>2</sup>		5	1120	599	1131	597	409	125	1135	534	494	107	985	513
	6	1080	573	1076	592	415	69	1087	536	490	61	956	524	
	10	1082	601	1078	567	419	92	1071	564	415	92	977	547	
	11	1091	602	1087	576	436	75	1037	540	398	99	967	529	
	12	1099	594	1095	593	458	44	1066	564	419	83	990	548	
Av.		1094	594	1093	585	427	81	1079	548	443	88	975	532	
III <sup>3</sup>	13	1096	610	1092	596	404	92	1039	539	366	134	988	534	
	14			1084	570	352	162	1019	547	363	109	976	513	
	15	1101	600	1093	588	424	81	1019	560	343	107	971	514	
	Av.		1098	605	1090	585	393	112	1023	549	357	117	978	520
	IV <sup>4</sup>	16	1096	593	1091	583	442	66	901	522	339	40	951	534
17		1094	595	1097	567	433	97	896	529	320	47	948	518	
18		1106	580	1100	565	419	116	905	500	322	83	952	505	
Av.			1099	589	1096	572	431	93	901	517	327	57	950	519
V <sup>5</sup>		19	1129	613	1118	605	421	92	928	534	301	93	950	527
	20	1139	635	1127	607	393	127	922	520	314	88	940	554	
	Av.		1134	624	1122	606	407	109	925	527	91	945	540	
	SUBJECT H													
	I <sup>1</sup>	7	1077	580	1077	601	388	88	1077	578	409	90	961	521
8		1089	576	1085	588	388	109	1085	540	448	97	982	535	
9		1114	588	1109	586	440	83	1109	566	427	116	998	548	
Av.			1093	581	1090	592	405	93	1090	561	428	101	980	535
II <sup>2</sup>		5	1120	599	1131	597	409	125	1135	534	494	107	985	513
	6	1080	573	1076	592	415	69	1087	536	490	61	956	524	
	10	1082	601	1078	567	419	92	1071	564	415	92	977	547	
	11	1091	602	1087	576	436	75	1037	540	398	99	967	529	
	12	1099	594	1095	593	458	44	1066	564	419	83	990	548	
Av.		1094	594	1093	585	427	81	1079	548	443	88	975	532	
III <sup>3</sup>	13	1096	610	1092	596	404	92	1039	539	366	134	988	534	
	14			1084	570	352	162	1019	547	363	109	976	513	
	15	1101	600	1093	588	424	81	1019	560	343	107	971	514	
	Av.		1098	605	1090	585	393	112	1023	549	357	117	978	520
	IV <sup>4</sup>	16	1096	593	1091	583	442	66	901	522	339	40	951	534
17		1094	595	1097	567	433	97	896	529	320	47	948	518	
18		1106	580	1100	565	419	116	905	500	322	83	952	505	
Av.			1099	589	1096	572	431	93	901	517	327	57	950	519
V <sup>5</sup>		19	1129	613	1118	605	421	92	928	534	301	93	950	527
	20	1139	635	1127	607	393	127	922	520	314	88	940	554	
	Av.		1134	624	1122	606	407	109	925	527	91	945	540	



Summaries of the phosphorus balances in mg/kg of body weight and of the percentages of phosphorus retained are given in table 2.

*Phosphorus intake and retention.* Phosphorus metabolism has received less attention than that of calcium and nitrogen, probably because the phosphorus needs are satisfied if the calcium and protein needs are adequately met through common foods. The National Research Council's recommended dietary allowances ('45) do not include values for phosphorus, but suggest that the phosphorus of children's diets should equal or exceed the calcium content. In this study these conditions have been met since the calcium:phosphorus ratios for the four children on the high-milk intake ranged from 0.86 to 0.91, and for the low-milk group from 0.69 to 0.82. The phosphorus intakes for the eight children ranged from 896 to 1374 mg, or from 55 to 76 mg/kg of body weight. These intakes are similar to those given in other reports on phosphorus metabolism of preschool children (Porter-Levin, '33, '33-'34; Daniels et al., '35, '37; Pierce et al., '40; and Hawks et al., '42).

Most of the reports of phosphorus retentions by preschool children are made in terms of mg/kg/day. Porter-Levin ('33-'34) reported average retentions of 5, 5, and 8 mg/kg; Daniels et al. ('35) 6-9 mg; Pierce et al. ('40), 6.9 to 7.4 mg/kg; and Hawks et al. ('42), 3 to 8 mg/kg. The average retentions for the eight subjects of this study ranged from 3.5 to 10.8 mg/kg on the various diets used.

Macy ('42) states that 48 to 65% of the ingested phosphorus is excreted in the urine, suggesting the wide use of phosphorus in metabolism. It also suggests that the supply of phosphorus to the tissues is generally ample, or smaller excretions would be found. The phosphorus excretions for the eight children in this study are in line with those reported in the literature, and indicate adequate supply even on the lower intakes.

*Effect of ascorbic acid supplement on phosphorus retention.* Daniels and Everson ('37) found no significant changes in phosphorus retention with variations in ascorbic acid intake,

TABLE 2  
*Summary of phosphorus balances of eight preschool children.*

DIET	INTAKE	EXCRETION		RETENTION	
		Urine	Feces		
	mg/kg	mg/kg	mg/kg	mg/kg	% of intake
Subject A					
I <sup>1</sup>	57	25	26	6.6	11
II <sup>2</sup>	57	26	26	5.9	10
III <sup>3</sup>	57	27	21	8.6	15
IV <sup>4</sup>	55	27	23	5.3	10
Subject B					
I <sup>1</sup>	76	43	25	7.9	10
II <sup>2</sup>	74	43	24	8.7	12
III <sup>3</sup>	73	43	26	4.1	6
IV <sup>4</sup>	71	41	24	6.5	9
Subject C					
I <sup>1</sup>	72	39	24	9.4	13
II <sup>2</sup>	70	40	24	6.2	9
III <sup>3</sup>	68	36	25	6.9	10
IV <sup>4</sup>	66	38	25	4.1	6
Subject D					
I <sup>1</sup>	75	45	24	6.3	8
II <sup>2</sup>	74	44	21	8.3	11
III <sup>3</sup>	71	40	20	10.6	15
IV <sup>4</sup>	69	41	19	8.4	12
Subject E					
I <sup>1</sup>	68	36	27	5.0	7
II <sup>2</sup>	68	37	27	4.1	6
III <sup>3</sup>	66	36	25	4.6	7
IV <sup>4</sup>	65	35	25	5.4	8
V <sup>5</sup>	66	36	22	7.3	11
Subject F					
I <sup>1</sup>	59	32	22	5.0	9
II <sup>2</sup>	58	31	23	4.3	7
III <sup>3</sup>	57	31	21	5.9	10
IV <sup>4</sup>	56	29	22	4.7	8
V <sup>5</sup>	57	31	21	5.5	10
Subject G					
I <sup>1</sup>	69	36	27	6.4	9
II <sup>2</sup>	68	34	28	5.5	8
III <sup>3</sup>	62	33	22	7.0	11
IV <sup>4</sup>	55	31	20	3.5	6
V <sup>5</sup>	56	32	19	5.5	10
Subject H					
I <sup>1</sup>	71	39	24	8.3	12
II <sup>2</sup>	71	39	27	5.1	7
III <sup>3</sup>	69	37	22	10.8	16
IV <sup>4</sup>	66	36	23	6.6	10
V <sup>5</sup>	66	37	21	6.9	10

<sup>1</sup> Basal diet only.

<sup>2</sup> Basal diet plus ascorbic acid.

<sup>3</sup> Basal diet plus potassium citrate.

<sup>4</sup> Basal diet plus ascorbic acid and potassium citrate.

<sup>5</sup> Basal diet plus orange juice.

but Shepherd et al. ('40) report decreases of 1.4 mg/kg/day on the addition of 20 mg of ascorbic acid. The decrease was ascribed to increases in the urinary excretion of phosphorus. In the eight children of the present study when 100 mg ascorbic acid were added, the phosphorus retentions were decreased for five, increased for one, and showed little difference for two of the subjects, but the differences in retention with and without the supplement were not significant for any child.

*Effect of potassium citrate supplement on phosphorus retention.* Comparison of the average retentions of phosphorus when 3.38 gm of potassium citrate were added to the diet, with the retentions without this supplement, shows that the phosphorus retentions were increased for four children, decreased for three, and unchanged for one with the supplement. The differences in retention were not significant for any child.

*Effect of orange juice supplement on phosphorus retention.* Approximately 218 ml of orange juice were added to the basal diet of subjects E, F, G, and H during the last 2 weeks of the study. The data for these periods are included in tables 1 and 2 under diet V. For subject E the retentions of phosphorus on this supplement were the greatest found at any time, but for the other three, the differences in retention were not appreciable. Chaney and Blunt ('25) have reported increased phosphorus retention in two adolescent girls; Daniels and Everson ('37) reported no significant differences in retention for three preschool children; and Shepherd et al. ('40) reported decreased retention in 5 young children when fresh orange juice was added to the diet. The results of the present study support the evidence of Daniels and Everson that there is no significant effect of addition of orange juice on the retention of phosphorus in preschool children.

#### SUMMARY AND CONCLUSIONS

Phosphorus metabolism has been studied in eight preschool children, on phosphorus intakes of 896 to 1374 mg/day, or

55 to 76 mg/kg/day. The values for retentions per kilogram of body weight were in line with those reported in the literature, average values varying from 3.5 to 10.8 mg/kg. The basal diet was supplemented with ascorbic acid, potassium citrate, and orange juice as indicated. None of the supplements caused significant alterations in phosphorus retentions, although rather wide variations occurred from period to period. This might indicate that other factors than diet are largely responsible for fluctuations in phosphorus retentions.

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# NITROGEN METABOLISM OF PRESCHOOL CHILDREN

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Studies by Daniels and Everson ('37) and Shepherd et al. ('40) present conflicting data on the effect of changes in the ascorbic acid content of the diet on the nitrogen retention by preschool children. This report presents data on the nitrogen metabolism of eight preschool children whose diet was supplemented with ascorbic acid, potassium citrate, and orange juice. The effect of these supplements on ascorbic acid, citric acid, calcium, and phosphorus metabolism has been reported in previous papers by Meyer and Hathaway ('44), Metcalf and Hathaway ('45), Watson and associates ('45), and McKey and associates ('46).

## EXPERIMENTAL

Two groups of four children each lived at the college laboratory apartment for 5 months. Subjects A, C, F, G, and H were girls, aged 55, 49, 49, 40, and 38 months, respectively, at the beginning of the study. Their respective weights at that time were 50, 38, 40, 32, and 29 pounds. Subjects B, D, and E were boys, aged 51, 44, and 55 months, and weighing 36, 35, and 34 pounds, respectively.

The general plan of the complete experiment, including a description of all subjects and of the diets used, has been reported in detail by Meyer ('43), and summarized in the previous papers of this series. The following facts are partic-

ularly pertinent to this study. The children were maintained on a basal diet adequate in all nutrients except ascorbic acid. The first group of children (A, B, C, and D) were given 800 ml of milk daily; the other group (E, F, G, and H) received only 500 ml. Through an increase in the meat, eggs, and peanut butter similar levels of protein were maintained in the basal diets of the two groups. Ry-Krisp or Toasted Wheat Wafers were allowed ad libitum so that there was some variation in nitrogen intake from period to period for a given child and from child to child for subjects A through F. Somewhat less of the basal foods was consumed by subject H throughout the study, and by subject G during the preliminary weeks and during periods 16 through 20, resulting in lower nitrogen intakes for these two girls. Supplements of crystalline ascorbic acid (100 mg), potassium citrate (3.38 gm) or both were given as indicated in table 1. These amounts correspond roughly to the amounts of ascorbic acid and of citrate-ion in 200 ml of orange juice. During the last 2 weeks, the low-milk group was given orange juice in place of the crystalline supplements.

The methods of collecting and preparing samples for analysis have been described by Metcalf and Hathaway ('45) and Watson et al. ('45). The nitrogen content of the foods and excreta was determined using the Hengar semi-micro Kjeldahl apparatus. The distillation tubes were equipped with water jackets (8 inches long and 3 inches in diameter) to cool the distillate and so prevent loss of ammonia.

#### RESULTS AND DISCUSSION

The data for this study are presented in table 1. Data for the preliminary adjustment periods (periods 1 and 4) have been omitted. A summary of the nitrogen balances in mg/kg of body weight is given in table 2.

*Nitrogen intake, absorption, and retention.* The protein allowance in the diets of these children was planned to meet the standards of Holt and Fales ('21) and was equal to or above the suggested allowances of the National Research

DIET PERIOD	SUBJECT A				SUBJECT B				SUBJECT C				SUBJECT D			
	INTAKE	EXCRETION	RETENTION	INTAKE	EXCRETION	RETENTION	INTAKE	EXCRETION	RETENTION	INTAKE	EXCRETION	RETENTION	INTAKE	EXCRETION	RETENTION	INTAKE
	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg
I <sup>1</sup>	5	9345	7659	883	803	762	969	8212	766	8905	8100	73	8790	7657	926	207
	6	9188	7512	923	753	9194	891	9309	800	9309	8100	409	9230	7488	874	868
	10	9148	7574	800	474	9118	949	9188	734	9188	7774	734	8949	7224	846	879
	11	9148	7574	800	474	8909	7425	889	595	9187	7896	709	8865	7228	734	903
	12	8762	7337	823	402	8567	7280	857	430	8735	7520	501	8567	7307	634	626
	Av.	9111	7046	857	608	8930	7445	911	583	9059	7900	745	8880	7381	803	697
II <sup>2</sup>	7	9033	7493	806	734	8873	7444	966	463	9033	7533	726	8829	7329	749	751
	8	9093	7436	891	766	9004	7339	883	782	9066	7601	752	8889	7502	554	833
	9	9002	7286	931	785	8923	7399	951	573	9020	7658	623	8816	7306	666	844
	13	8926	7605	777	544	8722	7456	883	383	8846	7525	694	8748	7354	583	811
	14	9020	7432	820	768	8719	7337	860	522	8869	7234	777				
	Av.	9015	7450	845	719	8848	7395	909	545	8967	7510	714	8820	7373	638	810
III <sup>3</sup>	15	8866	7229	846	791	8707	7089	766	852	8804	7189	651	8751	7151	631	969
	16	8725	7243	789	693	8530	7048	923	559	8636	7372	651	8539	7020	551	968
	17	8583	7054	846	683	8397	7220	906	271	8548	7327	794	8437	7307	846	779
	Av.	8725	7175	827	722	8545	7119	865	561	8663	7296	699	8574	7126	676	772
IV <sup>4</sup>	18	8723	7197	826	700	8537	7006	906	625	8670	7255	674	8546	6927	703	916
	19	8789	7237	800	752	8630	7265	917	448	8701	7440	869	8639	7111	766	762
	20	8840	7316	789	735					8804	7404	757	8733	7220	646	867
	Av.	8784	7250	805	729	8583	7135	911	536	8725	7366	767	8639	7086	705	848
DIET PERIOD	SUBJECT E				SUBJECT F				SUBJECT G				SUBJECT H			
	INTAKE	EXCRETION	RETENTION	INTAKE	EXCRETION	RETENTION	INTAKE	EXCRETION	RETENTION	INTAKE	EXCRETION	RETENTION	INTAKE	EXCRETION	RETENTION	INTAKE
	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg
I <sup>1</sup>	7	8506	7299	777	430	8506	7755	331	390	8506	6960	950	7340	6029	797	514
	8	8560	7280	800	390	8700	7700	631	369	8704	6835	1049	7419	6090	844	485
	9	8594	7280	924	390	8748	7986	546	206	8752	7072	1056	7441	6190	769	482
	Av.	8553	7286	864	403	8651	7857	503	322	8654	6956	1018	7400	6103	803	494
II <sup>2</sup>	5	8800	7496	893	335	8848	8086	560	202	8871	7056	1084	731	6114	951	460
	6	8728	7395	1070	263	8599	7929	546	124	8656	7059	1124	734	7382	6063	333
	10	8733	7210	917	606	8873	7793	506	574	8841	7041	1074	736	7595	6017	897
	11	8693	7344	876	473	8833	7801	506	526	8655	6779	1028	7548	5854	876	881
	12	8557	7198	854	505	8697	8006	563	128	8558	7071	1016	7541	6317	866	818
	Av.	8702	7329	937	436	8770	7923	536	311	8716	7001	1065	7500	6033	915	552
III <sup>3</sup>	13	8458	7706	820	—68	8598	7807	537	254	8343	6713	1011	7313	5991	911	411
	14	8532	7131	997	404	8667	7449	440	778	8358	6745	947	7386	5875	900	611
	15	8650	7282	857	511	8786	7606	569	611	8389	6755	894	7325	5975	800	550
	Av.	8547	7373	891	282	8684	7621	515	548	8363	6738	951	7341	5947	870	524
IV <sup>4</sup>	16	8626	7417	889	320	8770	7670	606	494	7208	6365	680	7359	6080	896	453
	17	8643	7347	847	449	8822	7594	574	654	7216	6135	631	7358	6009	849	500
	18	8604	7172	830	602	8773	7468	563	742	7165	6021	899	7288	5866	763	539
	Av.	8624	7312	855	457	8788	7577	581	630	7186	6173	737	7335	6025	813	497
V <sup>5</sup>	19	8633	7056	931	646	8756	7357	866	533	7199	5844	653	7257	5793	886	578
	20	8736	7101	894	739	8845	7132	871	842	7310	6151	644	7288	5852	737	699
	Av.	8684	7079	912	692	8800	7244	868	687	7254	5997	648	7272	5822	811	638



Council ('45), varying from 45 gm for the 3-year olds, to 58 gm for the 5-year olds.

Since chiefly meat and eggs were used to maintain the protein intake when the milk in the diet was decreased, the quality of the protein was not changed materially when the source was altered.

It is impossible to completely determine the availability of the protein in the diet, since there is no known analytical method for determining how much of the fecal nitrogen is of metabolic origin. The difference between the nitrogen intake and fecal nitrogen does give, however, a conservative estimate of the nitrogen available for body use. This may be considered as "absorbed" nitrogen, and in children usually represents approximately 90% of the ingested nitrogen (Macy, '42). Values for subjects A to H, respectively, were equivalent to 91, 90, 92, 92, 90, 94, 89, and 88% of the intake with standard deviations of  $\pm 0.3\%$  or less.

The average nitrogen retention values for the eight children of this study fall well within the range reported by Macy ('42),  $700 \pm 470$  mg for 4-year olds, and  $610 \pm 390$  mg for 5-year olds. Nitrogen intakes were similar for subjects A through F and for subject G on the first three diets, but the retentions varied widely, from — 73 to 969 mg per day for individual periods, or from 282 to 848 mg per day for the periods on a specific diet. Similar fluctuations have been reported by Macy et al. ('36). Although the intakes for subject H were lower than for the other children, the retention values were similar to theirs, ranging from 333 to 818 mg per day. After diet III, the basal food intake of subject G was cut, reducing the nitrogen intake about 1 gm per day. This lowering of the nitrogen intake reduced the nitrogen retention in the period immediately following the change, and the results for her will be omitted when data from diet IV are discussed.

According to Macy ('42) when reasonably constant amounts of high quality protein are ingested and absorbed, the level of protein intake is probably not the controlling factor in

nitrogen retention. The data for the children of this study support this view.

*Effect of ascorbic acid supplement on nitrogen retention.* Daniels and Everson ('37) found lower nitrogen retentions on a diet containing about 50 mg of ascorbic acid (including 30 mg of orange juice) than on larger intakes of ascorbic acid (part crystalline ascorbic acid and part from orange juice). Since other factors than ascorbic acid are added when orange juice is given, the increase in nitrogen retention should not be ascribed solely to the increase in ascorbic acid intake. In the subjects studied by Shepherd et al. ('40) the changes in nitrogen retention on the addition of 20 mg of ascorbic acid were not conclusive.

In the present study any effect of high levels of ascorbic acid on the nitrogen retention should be apparent in a comparison of the results obtained with diets I and II, and diets III and IV (table 1). The results were inconsistent: the nitrogen retentions were increased for subjects D and E and decreased for subject B, whether the ascorbic acid was added to the basal diet or to the basal diet plus potassium citrate. Values for subjects C and H were increased when the ascorbic acid was added to the basal diet, but decreased when it was added to the basal diet plus potassium citrate. Values for subject A were increased when ascorbic acid was added to the basal diet alone, but were practically unchanged when added to the basal diet plus potassium citrate; on the contrary those for subject F were increased only when the citrate was included. For any one child, comparison of the nitrogen retention values for all the periods when ascorbic acid was added (diets II and IV) with those when it was omitted (diets I and III) show no statistically significant differences.

From the results recorded above it appears that the addition of 100 mg of crystalline ascorbic acid to a basal diet containing about 25 mg of the vitamin does not consistently alter the nitrogen retention.

*Effect of potassium citrate supplement on nitrogen retention.* Increased retention of nitrogen was noted when the diet of infants was supplemented with salts of vegetable acids (Krause, '33) or with calcium citrate (Weber, '32). Davis ('35) found higher nitrogen retention on basic than on acidic diets by twelve children 7 to 12 years of age. Since the pH of the urines of the eight subjects in the present study was much more alkaline on the potassium citrate supplement, it seemed possible that the nitrogen retentions might have been increased by the addition of that salt. Individual variations in retention from period to period on the same supplement mask the effects of changes in supplement, so that variations because of addition of potassium citrate are not statistically significant for any child. The retentions for subject F, however, do seem notably higher with the supplement.

*Effect of orange juice supplement on nitrogen retention.* Approximately 218 ml of orange juice were added to the basal diet of subjects E, F, G, and H during the last 2 weeks of the study. The data for these periods are included in tables 1 and 2 under diet V. The average retentions of nitrogen on this supplement were the highest found during the study for subjects E, F, and H, and were higher for subject G than during any period on diet IV (basal diet supplemented with ascorbic acid plus potassium citrate), the only diet during which she received the same level of nitrogen intake. The percentage retentions of nitrogen were higher for all four children than on any other diet. These results confirm those of Shepherd et al. ('40) who found that fresh orange juice improved the nitrogen retention of young children to a greater extent than the addition of crystalline ascorbic acid and those of Chaney and Blunt ('25) who found increased nitrogen retention when orange juice was added to the diet of two older girls. They do not support the conclusion of Daniels and Everson ('37) that orange juice per se does not influence nitrogen retention.

Although the two periods during which orange juice was given in the present study are probably inadequate to estab-

TABLE 2

*Summary of nitrogen balances of eight preschool children.*

DIET	INTAKE	EXCRETION		RETENTION	
		Urine	Feces		
	mg/kg	mg/kg	mg/kg	mg/kg	% of intake
Subject A					
I <sup>1</sup>	403	338	38	27	6.7
II <sup>2</sup>	399	330	37	32	8.0
III <sup>3</sup>	381	313	36	32	8.3
IV <sup>4</sup>	382	315	35	32	8.3
Subject B					
I <sup>1</sup>	538	448	55	35	6.5
II <sup>2</sup>	533	445	55	33	6.2
III <sup>3</sup>	497	414	50	33	6.6
IV <sup>4</sup>	496	412	52	31	6.2
Subject C					
I <sup>1</sup>	506	441	42	23	4.6
II <sup>2</sup>	493	413	39	41	8.3
III <sup>3</sup>	458	386	37	35	7.7
IV <sup>4</sup>	462	390	41	31	6.8
Subject D					
I <sup>1</sup>	532	442	48	42	7.8
II <sup>2</sup>	519	434	38	48	9.2
III <sup>3</sup>	482	400	38	43	9.0
IV <sup>4</sup>	480	394	39	47	9.9
Subject E					
I <sup>1</sup>	532	453	54	25	4.7
II <sup>2</sup>	538	453	58	27	5.0
III <sup>3</sup>	514	444	54	6	3.3
IV <sup>4</sup>	505	429	50	27	5.3
V <sup>5</sup>	503	410	53	40	8.0
Subject F					
I <sup>1</sup>	466	422	27	17	3.7
II <sup>2</sup>	470	424	29	17	3.5
III <sup>3</sup>	454	398	27	20	6.3
IV <sup>4</sup>	447	386	30	32	7.2
V <sup>5</sup>	444	366	44	35	7.8
Subject G					
I <sup>1</sup>	548	440	64	43	7.9
II <sup>2</sup>	548	440	67	41	7.5
III <sup>3</sup>	505	407	57	41	8.1
IV <sup>4</sup>	436	374	45	17	4.0
V <sup>5</sup>	438	362	39	37	8.4
Subject H					
I <sup>1</sup>	535	441	58	36	6.7
II <sup>2</sup>	544	438	66	40	7.4
III <sup>3</sup>	521	422	62	37	7.1
IV <sup>4</sup>	511	420	56	35	6.8
V <sup>5</sup>	505	404	56	44	8.8

<sup>1</sup> Basal diet only.<sup>2</sup> Basal diet plus ascorbic acid.<sup>3</sup> Basal diet plus potassium citrate.<sup>4</sup> Basal diet plus ascorbic acid and potassium citrate.<sup>5</sup> Basal diet plus orange juice.

lish the effect of a supplement on the nitrogen retention, the results do indicate possible beneficial effects of orange juice.

#### SUMMARY AND CONCLUSIONS

Nitrogen metabolism has been studied in eight preschool children on protein intakes of between 45 and 58 gm per day. The basal diet was supplemented with ascorbic acid, potassium citrate, and orange juice as indicated. The results were as follows:

1. The nitrogen intake was probably not a factor influencing nitrogen retention, since from 88 to 94% of the ingested nitrogen was absorbed, and only 3.3 to 9.8% was retained.

2. The nitrogen retention was not significantly altered by the addition of 100 mg of ascorbic acid to a diet containing 23 to 25 mg of the vitamin.

3. The nitrogen retention was not significantly altered by the addition of 3.38 gm of potassium citrate to the diet.

4. The percentage retention of nitrogen was highest in all four subjects tested, when an orange juice supplement, equivalent in ascorbic acid and citrate value to the crystalline supplements, was added to the basal diet.

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# THE COMPARATIVE GROWTH-PROMOTING VALUE OF THE PROTEINS OF WHEAT GERM, CORN GERM, AND OF SOME OTHER PROTEIN FOODS OF PLANT AND ANIMAL ORIGIN <sup>1</sup>

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Food products of plant origin have recently received particular consideration as sources of dietary protein that may be used to supplement or extend protein foods of animal origin in times of shortage. Outstanding among these are soybeans, peanuts, cottonseed, yeast, and corn and wheat germs. As compared with the others, the grain germs have received little attention with respect to the nutritional value of their proteins. Osborne and Mendel ('19) showed that wheat germ is more efficient for the growth and maintenance of rats than the whole wheat kernel. In a recent study, Hove and Harrel ('43a) concluded from the results of feeding experiments with young rats that "the protein of wheat germ as the sole source of protein in the diet is of as high a nutritional quality as animal proteins such as casein, skim-milk powder, dried egg white, and beef muscle." In a second paper these authors ('43b) reported that wheat germ and skim-milk powder are about equally efficient in improving the nutritive value of wheat flour, and excel corn germ and soybean meal in this respect. The superiority of wheat germ when

<sup>1</sup> Some of the data in this paper were presented in abstract form in *Federation Proceedings*, 1945, vol. 4, p. 156.



compared with corn germ as a supplement to wheat flour has been cited by Stare and Hegsted ('44). They also found that the proteins of wheat germ, corn germ, and skim-milk powder are practically of equal value in maintaining nitrogen balance in the adult dog. In nitrogen balance studies with growing rats, Mitchell and Beadles ('44) found that the biological value of the protein of corn germ is as high as that of round beef, and is 85% as digestible. Compared with autoclaved soybean protein, corn germ protein was found to be about equally digestible, but the digested protein was more available in satisfying the requirements of maintenance and growth.

The superior quality of the proteins of wheat and corn germs has been amply shown by the above cited investigations. It seemed desirable, however, to develop more information relating to their values as compared with those of other foods of plant and animal origin that are highly regarded as sources of dietary protein. Defatted wheat and corn germs, prepared for human consumption and having excellent keeping qualities, are now available in considerable quantities. It has been estimated that the potential annual production of wheat germ exceeds 150 million pounds, and that 600 million pounds of defatted corn germ could be produced.

#### EXPERIMENTAL

The growth-promoting values of the proteins of wheat germ and corn germ were determined by feeding experiments with young albino rats. For comparison, similar experiments were conducted on some other sources of high quality plant and animal proteins, namely, soybean, peanut, and cottonseed flours, skim-milk powder, dried whole egg powder, and casein (table 1). These materials supplied the sole source of protein in diets made adequate with respect to the essential non-protein dietary factors. They were incorporated in the diets in quantities to supply protein at 10, 15, and 17.5% levels. The average net gain in weight of each group of animals over a period of 6 weeks and the average gain in weight per gm of

protein consumed were used as standards of comparison for evaluating the proteins of the materials studied.

All diets contained 2% cod liver oil and 4% salt mixture (Osborne and Mendel, '19). The remainder of each of the diets consisted of one of the protein sources listed in table 1, sufficient corn oil to adjust the total fat content of the diet to 8%, and sufficient dextrinized corn starch to adjust the protein content to 10, 15, or 17.5%. A vitamin mixture was incorporated

TABLE 1

*Composition of the protein foods.*

*The percentages are calculated on an air-dry basis.<sup>1</sup>*

SOURCE	PROTEIN	FAT	SOURCE	PROTEIN	FAT
	%	%		%	%
Dried whole egg powder	46.00	34.27	Peanut flour	52.36	7.40
Skim-milk powder	37.31		Corn germ <sup>2</sup>	21.31	2.16
Soybean flour	46.73	6.47	Wheat germ <sup>2</sup>	34.30	0.40
Cottonseed flour	49.85	7.01	Casein <sup>3</sup>	89.70	.

<sup>1</sup> The following conversion factors were used in calculating the amounts of protein in the materials from their nitrogen content: Egg powder, 6.25; skim-milk powder, 6.38; soybean flour, 5.71; cottonseed flour, 5.30; peanut flour, 5.46; corn germ, 6.25; wheat germ, 5.80; casein, 6.38 (Jones, '31).

<sup>2</sup> Products defatted by solvent extraction at low temperature. Prepared by the Viobin Corporation, Monticello, Illinois.

<sup>3</sup> Labco.

ated in the dextrinized corn starch, which provided the following constituents in each 100 gm of diet: Thiamine hydrochloride, 0.2 mg; pyridoxine hydrochloride, 0.2 mg; riboflavin, 0.3 mg; calcium pantothenate, 0.3 mg; niacin, 1 mg; and choline hydrochloride, 10 mg. These vitamins were added to the dextrinized starch in an aqueous-alcoholic solution and the mixture was dried at 50°C. to the original weight of the starch.

The data presented represent results obtained with forty-nine lots of albino rats comparable in all respects. Forty-one of these lots were fed ad libitum and the remainder by the paired feeding method. Most of the lots consisted each of twelve animals (in some cases eight animals were used),

equally divided with respect to sex and having initial weights of 45 to 60 gm. They were housed in individual cages having wide mesh screen bottoms and kept in an air-conditioned room maintained at 76°F. ( $\pm 2^\circ\text{F}.$ ) and about 55% relative humidity. The animals on the ad libitum experiments were weighed and fed twice weekly. Fresh diets were prepared in kilogram quantities each week.

The paired feeding experiments were carried out as follows: Eight pairs of albino rats were used in each experiment. Each pair was of the same sex, from the same litter, and the pair mates did not differ in initial weight by more than 5 gm. The initial weights between pairs ranged from 45 to 60 gm. Both animals of a pair received a diet similar in all respects with the exception of the source of protein. One animal of a pair was fed ad libitum the diet that was consumed in the smaller quantity. The amount of food consumed by the other animal was determined by the quantity of food the rat fed ad libitum had consumed the previous day. In this way, the total food intake of both rats of a pair was practically the same at the end of the feeding period.

#### RESULTS AND DISCUSSION

As determined by the ad libitum method of feeding, wheat germ as a source of protein showed definitely higher nutritive values than corn germ at each of the three different levels of protein fed when calculated either on the net weight gains of the animals or on their gains per gm of protein consumed (table 2). This was especially pronounced at the 10% protein level.

The values of corn germ as compared with the oilseed flours varied with the different protein levels at which the materials were fed, when measured either by gains in body weight or by gains per gm of protein consumed. At a 10% protein level corn germ gave higher values than those similarly obtained with the oilseed flours when measured by gains in body weight. When measured in terms of weight gains per gm of protein consumed at a 10% level, soybean flour gave a higher value

than did corn germ. The comparative nutritive value of the plant protein studied at the 10% protein level on the basis of weight gains ranges from the highest downward in the following order: Wheat germ, corn germ, soybean flour, cottonseed flour, peanut flour. On the basis of gain in weight per gm of protein consumed they range in the same order,

TABLE 2

*Comparative growth-promoting values of the proteins of wheat and corn germs, and of other protein foods, when fed as the sole source of protein in the diet. (Tests conducted by the ad libitum method of feeding over a period of 6 weeks.)*

SOURCE OF PROTEIN	AVERAGE WEIGHT GAIN	STANDARD DEVIATION	AVERAGE GAIN/100 PROTEIN CONSUMED	STANDARD DEVIATION	AVERAGE FOOD CONSUMED
	gm		gm		gm
10% Protein in diet					
Wheat germ	132	± 29.12	2.54	± 0.190	518
Corn germ	101	± 15.83	2.11	± 0.178	478
Soybean flour	95	± 21.14	2.32	± 0.238	407
Cottonseed flour	82	± 12.38	1.89	± 0.162	431
Peanut flour	63	± 15.66	1.82	± 0.299	341
Whole egg powder	180	± 47.42	3.25	± 0.518	547
Skim-milk powder	130	± 15.87	2.64	± 0.170	492
Casein	120	± 14.68	2.41	± 0.225	497
15% Protein in diet					
Wheat germ	140	± 34.19	1.84	± 0.201	504
Corn germ	114	± 30.61	1.56	± 0.245	481
Soybean flour	104	± 21.28	1.49	± 0.216	462
Cottonseed flour	132	± 20.44	1.80	± 0.137	489
Peanut flour	102	± 19.60	1.69	± 0.207	400
Whole egg powder	175	± 39.24	2.35	± 0.292	493
Skim-milk powder	161	± 37.86	2.05	± 0.234	518
Casein	135	± 35.61	1.74	± 0.241	510
17.5% Protein in diet					
Wheat germ	131	± 35.67	1.58	± 0.251	467
Corn germ	112	± 23.71	1.29	± 0.171	491
Soybean flour	95	± 24.53	1.21	± 0.189	446
Cottonseed flour	132	± 20.96	1.64	± 0.182	460
Peanut flour	114	± 25.61	1.53	± 0.205	426
Whole egg powder	121	± 40.86	1.59	± 0.295	426
Skim-milk powder	144	± 26.98	1.82	± 0.203	452
Casein	174	± 27.65	1.90	± 0.231	524

with the exception of corn germ and soybean flour, which are reversed.

At the higher protein levels (15 and 17.5%) quite a different order of values was obtained. At the 15% level, wheat germ still held first place among the plant proteins both with respect to average weight gain and gain per gm of protein consumed. Cottonseed flour, however, ranked second. Corn germ came next in the order of values with respect to average weight gain, and soybean and peanut flours showed practically the same values. With respect to gain per gm of protein consumed peanut flour ranked third, with corn germ and soybean flour following as indicated in the table.

At the 17.5% protein level wheat germ and cottonseed flour gave practically the same values as indicated both by weight increases and gain per gm of protein consumed. Peanut flour, corn germ, and soybean flour followed in the order named.

When compared at the different protein levels in the diet, the animal proteins proved, in general, superior to the plant proteins. When fed at the 10% protein level dried egg powder gave the highest value obtained at any level with any of the proteins studied. At this level wheat germ and skim-milk powder gave essentially the same values with respect to weight gains and gains per gm of protein consumed, and both proved better than casein. With 15% protein in the diet, the highest values were obtained with egg powder. Skim-milk powder, wheat germ, and casein followed in the order named. At the 17.5% level casein gave the highest value. The low values found for the egg powder as compared with those obtained at the 10% and 15% protein levels are probably to be ascribed to a poorer quality product, the use of which was made necessary because of depletion of the original supply.

The values found for the corn germ and soybean flour proteins by the paired feeding method (table 3) are, in general, in fairly close agreement with those obtained by the ad libitum method (table 2). With wheat germ and dried egg powder, however, lower values were obtained by the paired feeding method. The nutritive values of the protein foods com-

pared, both with respect to body weight gains and gains per gm of protein consumed, were found in general to rank in the same descending order when determined by both procedures, namely, egg powder, wheat germ, corn germ, soybean flour. However, when measured by average gain in weight per gm of protein consumed the values for corn germ and soybean flour were essentially the same. In these experiments the materials studied were incorporated in the diets at a 10% protein level.

TABLE 3

*Paired feeding tests.*

*Averages of results obtained with 8 pairs of rats used in each experiment. Ten per cent protein level in the diets. Duration of feeding tests — 6 weeks.*

MATERIAL	AVERAGE BODY WEIGHTS		AVERAGE FOOD CONSUMPTION	AVERAGE	
	Initial	Final		Weight gains	Gain per gram of protein and standard deviation
	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>
Wheat germ	51	166	463	115	2.47 $\pm$ 0.175
Corn germ	53	154	465	101	2.18 $\pm$ 0.230
Wheat germ	53	172	442	119	2.69 $\pm$ 0.150
Soybean flour	55	150	430	95	2.20 $\pm$ 0.174
Corn germ	50	152	423	102	2.41 $\pm$ 0.207
Soybean flour	49	141	410	92	2.25 $\pm$ 0.237
Wheat germ	51	166	489	112	2.35 $\pm$ 0.253
Egg powder	52	194	490	142	2.91 $\pm$ 0.416

Hove and Harrel ('43b) reported that "a fair percentage of their rats fed wheat germ at 10% protein level in the diet developed a severe hemorrhagic disease characterized by sudden onset, marked weight loss, severe anemia, and in most cases death within four days." This difficulty did not occur when 1% of liver-extract powder was included in the ration. At none of the protein levels used in our rations was any adverse effect observed with either wheat germ or corn germ.

A statistical analysis was made to test the significance of the differences between the average weight gains obtained by the ad libitum method of feeding for the various protein sources. The weight gains for wheat germ and corn germ

were compared, first with one another, and then with each of the remaining proteins used in the experiment.

The highest degree of significance was found at the 10% level of protein in the diet. The differences in weight gain between wheat germ and all the other proteins, with the exception of skim-milk powder and casein, were found to be highly significant, while those for corn germ compared with the other sources were highly significant in all cases except soybean flour.

At the 15% level the significance of the differences was not so pronounced. A high degree of significance remained between weight gains for corn germ and those for whole egg and skim-milk powders, but the difference between the corn and wheat germs was only slightly significant. The differences between corn germ and the remaining proteins showed no significance. In the case of the wheat germ comparisons, the difference in gain from cottonseed flour was no longer significant, while peanut flour and soybean flour remained highly significant. Whole egg powder still showed a significant difference but to a lesser degree than at the 10% level.

Wheat germ was found to be significantly different only from soybean flour and casein at the 17.5% level, while corn germ differed significantly from cottonseed flour, skim-milk powder, and casein. The degree of significance for this level of protein was less in most cases than for the 10 and 15% levels.

It is of interest to note the downward slope of significance in the values with increasing protein levels in the diet above 10%.

#### SUMMARY

Growth-promoting values of the proteins of wheat germ and corn germ are reported. For comparison, values similarly obtained are given for several other protein foods of plant and animal origin — soybean, peanut, and cottonseed flours, dried whole egg powder, dried skim-milk powder, and casein.

The materials were fed as the sole source of protein to weanling rats at 10, 15, and 17.5% protein levels in diets approximately isocaloric and nutritionally adequate with respect to dietary factors other than protein. Both the ad libitum and paired feeding methods were used and the results were evaluated on the basis of weight increases and gains per gm of protein consumed over 6-week periods.

Consistently higher nutritional values were found for wheat germ than for corn germ at each of the different protein levels fed. Wheat germ also proved superior to the oilseed flours, and at the 10% level it was found as efficient as skim-milk powder, and more efficient than casein.

In general, the protein values of the materials determined by both the ad libitum and paired feeding methods were found to rank in the same descending order, namely, egg powder, wheat germ, corn germ, soybean flour.

Corn germ fed at 10 and 15% protein levels produced equal or greater weight gains than were obtained with peanut or soybean flours, but it was not as efficient as cottonseed flour when fed at 15 and 17.5% protein levels.

Definitely higher values at the 10 and 15% protein levels were obtained with the whole egg powder than with any of the other materials studied, but it was excelled by casein at the 17.5% protein level.

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# A STUDY OF PYRIDOXINE AND PANTOTHENIC ACID DEFICIENCIES IN THE MONKEY (MACACA MULATTA)<sup>1</sup>

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THREE FIGURES

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Previous studies (Waisman and Elvehjem, '43; Waisman, Rasmussen, Elvehjem and Clark, '43) have demonstrated that the rhesus monkey (*Macaca mulatta*) grows normally and remains in good health when fed purified diets containing the known vitamins and a small amount of a liver fraction or a "folic acid" concentrate. Through the use of this diet specific vitamin deficiencies have been studied by withholding the vitamin concerned from the daily supplement (Waisman, '44; Waisman and McCall, '44; Waisman, McCall and Elvehjem, '45; Cooperman, Waisman, McCall and Elvehjem, '45).

The importance of pyridoxine and pantothenic acid in the nutrition of various laboratory animals has been studied by many workers. Wintrobe, Follis, Miller et al. ('43) have reviewed the early literature dealing with the production of pyridoxine deficiency and have presented data on the effects of vitamin B<sub>6</sub> deficiency in swine. These workers have

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demonstrated a microcytic anemia and an increase of polychromatophiles, reticulocytes and nucleated red cells in the blood and a remission after the administration of vitamin B<sub>6</sub>. Pantothenic acid deficiency has been produced in the mouse (György and Poling, '40), rat (Henderson et al., '42), chick (Waisman et al., '39), dog (Schaefer et al., '42), and pig (Wintrobe et al., '42) but the manifestations of the deficiency have been somewhat different in the various species. An account of the deficiency of pantothenic acid in swine has been reported by Wintrobe, Follis, Alcayaga et al. ('43). Impairment of growth, diarrhea, anorexia, unkempt hair, alopecia, and slight edema were the symptoms observed. Histological evidence demonstrated injury to the cells lining the glands of the colon. Blood studies have revealed a moderate normocytic anemia with a decrease in the plasma chlorides. Non-protein nitrogen increased only in extreme deficiency and may be due to renal failure.

In this paper we wish to describe pyridoxine and pantothenic acid deficiencies in monkeys as seen in this laboratory, the responses to synthetic pyridoxine and pantothenic acid, and the concurrent deficiency of the monkey anti-anemic factor found in liver, previously described by Cooperman, Waisman, McCall and Elvehjem ('45).

#### EXPERIMENTAL PROCEDURES

Young immature rhesus monkeys weighing approximately 1.5-2.0 K were used in all the studies. The methods of handling and feeding the animals have been described previously (Waisman, Rasmussen, Elvehjem and Clark, '43). The basal diet (M-3), consisting of sucrose 73 parts, purified casein 18, mineral salts 4, cod liver oil 3, corn oil 2, was fed ad libitum; and adequate quantities of ascorbic acid, thiamine, riboflavin, nicotinic acid, choline chloride, p-aminobenzoic acid, inositol, biotin, and pyridoxine or calcium pantothenate were given daily. In addition, a "folic acid" concentrate (Hutchings et al., '41) equivalent to 5 gm of solubilized liver powder (fraction L) was fed daily; this quantity was increased as the

experiments progressed to an equivalent of 10 gm of liver. The "folic acid" concentrate fed in the pantothenic acid studies was autoclaved at pH 10 for 30 minutes at 15 pounds pressure to destroy the pantothenic acid in the concentrate. Microbiological assays on the concentrate before and after this process showed that there was no loss of "folic acid" activity. After autoclaving, this material was cooled and neutralized before being fed.

The heated grain ration (241 H) of Kline et al. ('32) was also used for the production of pantothenic acid deficiency. It is low in pantothenic acid and is composed of yellow corn 58, wheat middlings 25, crude casein 12 parts which were mixed and heated for 30 hours at 120°C., salts IV 4, cod liver oil 3 and corn oil 2. It had been used extensively in the production of pantothenic acid deficiency in chicks (Waisman et al., '39) and rats (Henderson et al., '42), but had not previously been used for monkeys. This diet was supplemented daily with the same vitamin mixture that was used when the sucrose-casein basal (M-3) diet was fed.

Complete blood examinations including differential white cell counts (using Wright's stain) were made usually at weekly intervals throughout the period of depletion, deficiency and recovery. Hemoglobin was determined in the Evelyn photoelectric colorimeter and red and white cell counts were made in the usual manner by drawing blood from the marginal veins of the ear. Blood samples for chemical analysis were removed from the saphenous vein of the leg. The blood sugar, non-protein nitrogen and chlorides were determined on the Folin and Wu tungstic acid filtrates of the freshly drawn venous blood. Blood sugar was determined by the method of Benedict ('28), chlorides by the method of Whitehorn ('20-'21) and non-protein nitrogen by the method of Folin and Wu ('19).

#### PYRIDOXINE STUDIES

Four young monkeys (nos. 136, 137, 138, 139) were placed on the M-3 basal ration with the norite eluate concentrate plus all of the crystalline B vitamins except pyridoxine. All

four animals failed to grow normally and generally maintained their initial body weight with little variation for a period of about 9 months, when a marked loss of weight was observed in three of the animals. Test doses (1 mg per day) of pyridoxine were given for 7 days to two animals (nos. 136, 137) after they had been on this regimen for 6 months to determine if a pyridoxine deficiency was developing. Both animals showed immediate blood responses and weight gains

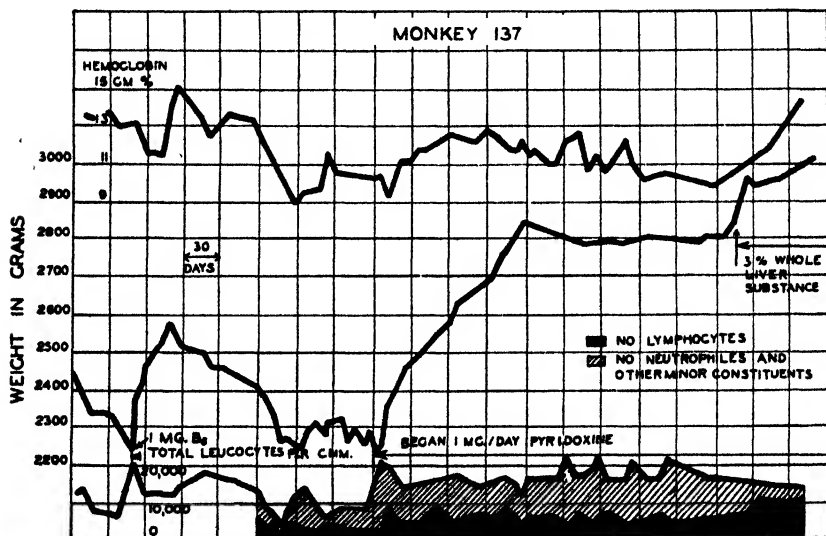


Fig. 1 Effect of pyridoxine deficiency on weight, hemoglobin, total and differential leucocyte count of a typical monkey, and the response to pyridoxine and whole liver substance.

as long as the pyridoxine was continued. The increased body weight was maintained for 3 months before it decreased to the same level as that before the administration of pyridoxine (fig. 1).

During this period of depletion, a hypochromic microcytic anemia developed in all of the animals and nucleated erythrocytes appeared in the blood. Hemoglobin values as low as 8 gm % were observed while the erythrocyte count decreased only slightly (4.5–5.5 million per mm<sup>3</sup>). All four of

the animals at this time demonstrated marked inactivity, general weakness, hyperirritability and poor appetite, and had little interest in the vitamin supplement or the dry ration, although all of the supplement and a substantial amount of the ration were consumed during the day. All of the animals showed thinning of the fur and some greying of the fur on the legs, arms and back. However, the greying never progressed to a marked extent.

All four showed some polychromatophilia and this condition was more marked in two animals (nos. 136, 137). The total white cell count decreased, but the changes were so variable that no distinct pattern in the total count in relation to the anemia was discernible. However, the differential leucocyte count revealed an increase in the percentage of neutrophiles and a proportionate decrease in the percentage of lymphocytes. The neutrophiles increased to 55-60% of the total leucocytes, whereas the normal value is about 36% (Shukers et al., '38). At the same time the lymphocytes decreased from a normal of 59% to 25-35% of the total leucocytes. There was no significant change from the normal in the percentage of basophiles or eosinophiles and monocytes.

Within 2 weeks after 1 mg of pyridoxine per day was given to three of the monkeys (nos. 137, 138, 139), the animals began to gain weight at a normal rate, the hemoglobin values showed an increase and the polychromatophiles decreased. Nucleated red blood cells were no longer observed in blood. With the pyridoxine therapy the neutrophile-lymphocyte ratio tended to return to normal but the improvement was temporary and within 2 to 3 months the reversal was as marked as that preceding pyridoxine additions. Complete blood regeneration resulted within 5 weeks after whole liver substance was added at a level of 3% to the ration of 2 of these animals.

Monkey 136 was continued on the pyridoxine-deficient diet and slowly began to lose weight about 5 months after the other three animals first showed marked loss of weight. Since at this time the weight losses and anemia were not critical,

pyridoxine therapy (1 mg per day) was delayed for 3 months, during which time a rapid loss of weight occurred. Significant growth and hemoglobin responses occurred when pyridoxine was given, but the animal developed dysentery and died after 4 weeks.

It is significant that whole liver substances maintained the hemoglobin level at a value considerably higher than the published normal value of 12.2 gm % (Shukers et al., '38). These high values in the whole liver-treated monkeys, i.e., 14.5–15.5 gm % of hemoglobin, have been repeatedly observed in the monkeys in this laboratory which have received fresh or lyophilized liver (Cooperman, McCall and Elvehjem, '45) or whole liver substance.

#### PANTOTHENIC ACID STUDIES

##### *Studies with the heated ration*

The heated ration as described was fed to four monkeys (nos. 104, 105, 117, 118). Two of these (nos. 104, 105) served as controls and received 3 mg daily of calcium pantothenate. All of them grew slowly, but the two controls grew slightly better than the animals fed the heated ration with no added calcium pantothenate. After 6 months, it was observed that the deficient group showed poorer hair color, although the quantity of fur was apparently unaffected. Although the four animals were continued on this diet for 9 months, no significant differences in blood sugar, non-protein nitrogen, serum chlorides or serum proteins were observed. Since Schaefer et al. ('42) had reported hypoglycemia, increased non-protein nitrogen and a drop of serum chlorides in pantothenic acid-deficient dogs, it was apparent that pantothenic acid deficiency in the dog on the "synthetic diet" and in the monkey on the heated grain ration are not strictly comparable.

##### *Studies with the synthetic ration*

After 9 months the deficient animals (nos. 117, 118) were placed on the synthetic M-3 basal ration and given all the

B vitamins except calcium pantothenate. At the same time, five young monkeys were also placed on this regimen. The significant data for two of these monkeys (112 and 118) are given in figures 2 and 3 and a rather detailed description of the deficiency syndrome is included for monkey 112.

*Monkey 112 ♂ (fig. 2).* No increase in body weight occurred during the first 5 weeks and then the animal started to lose weight slowly without showing any gross symptoms.

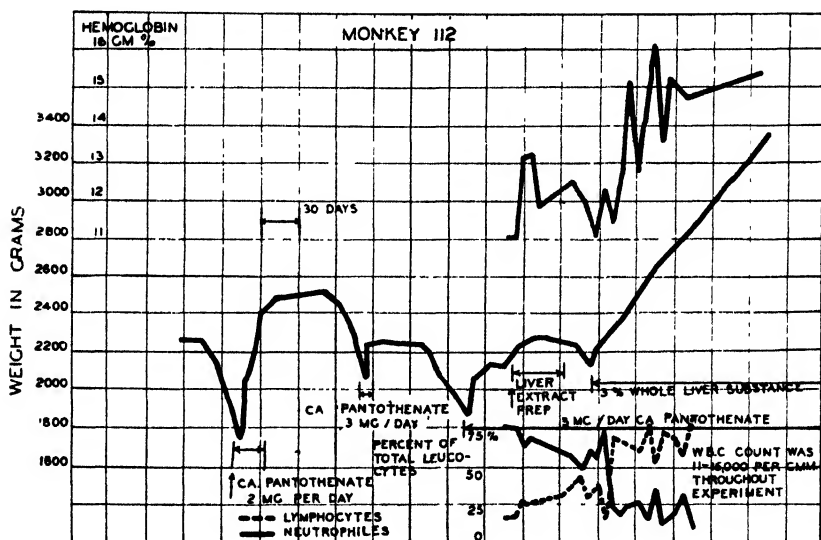


Fig. 2 Effect of pantothenic acid and liver factor deficiency on weight, hemoglobin, neutrophile and lymphocyte count of monkey no. 112 and the responses to calcium pantothenate, liver extract and whole liver substance.

After 4 weeks of continued loss in weight, 1 mg per day of calcium pantothenate was given and an immediate weight response resulted. This supplement was discontinued after 9 weeks. During the following 9-week period the animal gradually lost its fur, and became quite denuded and finally began to lose weight again. Following a weight loss of about 500 gm the biotin supplement was increased to 40  $\mu$ g daily, but this change did not prevent a further weight loss. At this point 2 mg of calcium pantothenate per day were given and



continued for 16 days. Again a rapid weight response was obtained and new hair appeared after 2 weeks, although the new fur coat was not very heavy. Eight weeks after calcium pantothenate was discontinued the animal began to lose weight again and 3 mg of calcium pantothenate were given daily for 8 days. This treatment produced a weight gain, but during the 7 weeks following the removal of all the calcium pantothenate from the daily supplement, greying of the fur

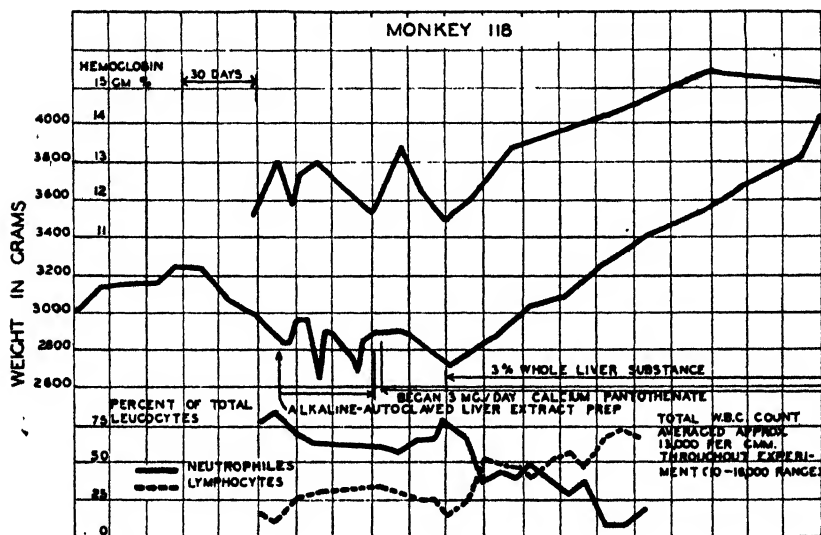


Fig. 3 Effect of pantothenic acid and liver factor deficiency on weight, hemoglobin, neutrophile and lymphocyte count of monkey no. 118 and the responses to a liver extract preparation, calcium pantothenate and whole liver substance.

and loss in weight resulted. The animal appeared emaciated, had unkempt fur, and showed evidence of muscular weakness in the legs. The blood picture indicated a mild anemia and a reversal of the neutrophile-lymphocyte ratio. The administration at this time of 3 mg of calcium pantothenate daily prevented further loss of body weight, but caused no general improvement in the condition; in fact, a large (3 cm) open sore developed on the bottom heel of the right foot and persisted for over 2 months in spite of treatment with

sulfathiazole applied topically. Monkey 122 developed similar sores on both feet that did not respond to either sulfathiazole or penicillin therapy.

After 3 months on this regimen, including 3 mg of calcium pantothenate daily, with no noticeable improvement, 3% whole liver substance was then added to the ration. Within 1 week the animal began to gain weight and became more active. The neutrophile-lymphocyte ratio returned to normal in 3 weeks and within 4 weeks the hemoglobin increased to 15.2 gm %. The animal was continued on this diet for 6½ months during which time the gain in weight was normal. Apparently a concurrent deficiency of factor(s) in whole liver required for complete blood regeneration also develops in pantothenic acid deficiency.

*Monkey 118 ♂.* Diarrhea and greying of the fur were observed about the time that the plateau in weight occurred. Four weeks later a pink rash appeared in the groin; this developed into an extensive dermatitis and scaliness and spread onto the inner areas of the calf and thigh and onto the inner side of the fore and upper arms. Dryness of the skin about the nose and unkempt fur were also seen. Definite improvement of the dermatitis occurred within 3 weeks after giving 3 mg per day of calcium pantothenate. At this time the ration was supplemented with 3% whole liver substance. Within 2 weeks, the monkey began to gain weight at a normal rate, the neutrophile-lymphocyte ratio and the hemoglobin returned to almost normal values. The whole liver substance was continued for 6 months and during this period the fur and the blood picture returned to normal.

In general the case histories of the other animals are similar to those described for monkeys 112 and 118.

#### DISCUSSION

It is evident from these experiments that monkeys given the purified basal ration with all the B-vitamins except pyridoxine develop a condition characterized by a hypochromic microcytic anemia, mild leucopenia, polychromatophilia,

presence of normoblasts, lack of growth, poor appetite and ataxia. In most respects these symptoms are similar to those observed in swine (Wintrobe, Follis, Miller et al., '43) and in dogs (McKibbin et al., '42).

Failure to produce a complete remission from the anemia with pyridoxine therapy has been observed both in dogs (McKibbin et al., '42) and in swine (Wintrobe, Follis, Miller et al., '43). A liver extract fraction (treated to remove the pyridoxine) was included in the ration fed to dogs, but these workers did not supply biotin, para-aminobenzoic acid or inositol. Smith et al. ('43) likewise have reported a typical hypochromic anemia in dogs which responded specifically to vitamin B<sub>6</sub> treatment, but brewers yeast at a level of 10% was required in addition to vitamin B<sub>6</sub> for complete blood regeneration.

Our results with pyridoxine deficient monkeys indicate that the additional factor is undoubtedly identical with the monkey anti-anemic factor required by riboflavin deficient monkeys to obtain complete blood regeneration. Differential white cell counts of riboflavin-deficient monkeys following the incomplete remission of the anemia by the administration of riboflavin reveal the same reversal of the neutrophile and lymphocyte count (unpublished data).

The pantothenic acid deficiency syndrome in monkeys is characterized by thinning and greying of the fur, lack of growth, extreme lassitude, emaciation, diarrhea, cachexia, ataxia and anemia. Follis and Wintrobe ('45) have demonstrated the role of pyridoxine and pantothenic acid deficiency in the degeneration of various portions of the nervous system (swine), and this action may account for the ataxia observed in our animals. The blood changes other than the severe anemia appear to be due to the concurrent deficiency of the factor(s) in whole liver substance. Monkey 117 that died on the pantothenic acid-low ration had a fatty and mottled liver and the adrenals were hemorrhagic, but no other gross pathological condition was observed.

It is significant that the blood picture which develops following calcium pantothenate therapy in the pantothenic acid deficient monkeys is identical with that which develops following incomplete remission of the anemia due to pyridoxine deficiency. Apparently this factor becomes limiting only after the pyridoxine or pantothenic acid is supplied following long depletion periods and with the resumption of growth. This would account for the short duration of the rapid growth responses following the administration of pyridoxine or pantothenic acid and the incomplete blood regeneration. Fresh or lyophilized liver (Cooperman, McCall and Elvehjem, '45) or whole liver substance supply the other factor(s) necessary to produce normal growth and concentration of blood constituents. Our data on the inactivity of 3% 1 : 20 liver extract powder in the prevention of this condition is further proof that the factor(s) is similar to that required to obtain complete blood regeneration and normal growth in riboflavin-deficient monkeys.

#### SUMMARY

Pyridoxine deficiency in young monkeys resulted when a synthetic diet was fed with all the crystalline vitamin B complex except pyridoxine. The symptoms were lack of growth, ataxia, hypochromic microcytic anemia, mild leucopenia, polychromatophilia and the appearance of nucleated red blood cells. With the administration of pyridoxine, growth was resumed and the anemia and blood picture improved.

A concomitant deficiency of the monkey anti-anemia factor occurred and was manifest by anemia and a reversal of the normal neutrophile/lymphocyte ratio. Whole liver substance at a level of 3% was necessary for optimum growth and blood regeneration.

Pantothenic acid deficiency syndrome was characterized by lack of growth, ataxia, greying and thinning of the fur, anemia, diarrhea and cachexia. Incomplete remission of these symptoms occurred on the administration of calcium panto-

thenate but complete recovery resulted when whole liver substance at a level of 3% of the ration was given.

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## COVITAMIN STUDIES

### V. THE INTERRELATION OF ALPHA-TOCOPHEROL AND ESSENTIAL UNSATURATED FAT ACIDS <sup>1</sup>

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THREE FIGURES

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The protection of vitamin A and carotene by tocopherols in vivo was the subject of the previous papers in this series (Hickman et al., '44; Harris et al., '44; Jensen et al., '43).

Destruction of carotene in the stomach of the rat requires the simultaneous presence of highly unsaturated fat acids. This was shown by Sherman ('41a) who also noted ('41b) that alpha-tocopherol prevented this destruction, in vivo. Peroxide formation at the double bonds of the fat acids probably occurs, with coupled oxidation (Sumner, '42) of carotene or vitamin A. Tocopherol protects the unsaturated bonds thus indirectly preserving the vitamin A. The oxidizing agent in the stomach responsible has been shown by Hove ('43) to be a water-soluble material similar to soybean lipoxidase.

Presumably a similar peroxidation of double bonds is responsible for the markedly increased vitamin E requirements when highly unsaturated fats are fed (Dam, '44; MacKenzie et al., '41).

If oxidation of the double bonds proceeds to a sufficient degree and extent, low levels of pure linolate should be less

<sup>1</sup> Communication No. 84 from the Laboratories of Distillation Products, Inc. A preliminary report of this work was published in Fed. Proc., vol. 4, March, 1945.



curative than the same levels with added  $\alpha$ -tocopherol or other fat-soluble antioxidant for rats deficient in the essential unsaturated fat acids. This possibility has been tested and the results, which show that this is an actuality, are reported here.

#### EXPERIMENTAL

##### *Diet and supplements*

A basal fat-free diet, indicated as diet 60, was made up with the following percentage composition: vitamin-free casein <sup>2</sup> 20; sucrose 76; salt mixture (U.S.P. XI, No. 2, plus  $\text{ZnCO}_3$ , 0.1%;  $\text{CuSO}_4 \cdot 7 \text{H}_2\text{O}$ , 0.08%;  $\text{MnSO}_4$ , 0.02%) 4. To this was added the following vitamins and related substances, in micrograms per gram of ration: thiamine hydrochloride 10, pyridoxine hydrochloride 10, riboflavin 10, calcium pantothenate 25, i-inositol 100, and choline chloride 1,000.

Vitamins A and D were fed weekly in the form of one drop (28 mg) of a fish oil concentrate, fortified with Delsterol, to furnish 2,000 units of A and 500 units of D per rat. In a few experiments crystalline carotene <sup>3</sup> and Delsterol were dissolved in coconut oil and one drop given daily to furnish 30 units of A and 10 units of D per rat. The synthetic water-soluble vitamins were thoroughly ground up with a small quantity of the casein, and this concentrate intimately mixed into the rest of the casein.

Methyl linolate was prepared every 10 days by the method indicated by Sherman ('41a). It was fed orally from a special capillary dropper calibrated to deliver exactly 5 mg per drop. The tocopherols were dissolved in propylene glycol and fed orally from droppers calibrated so that two drops were equivalent to 0.5 mg of the tocopherol.<sup>4</sup> The supplements were kept refrigerated in glass stoppered containers and flushed with nitrogen after each opening.

<sup>2</sup> Labco.

<sup>3</sup> Smaco.

<sup>4</sup> The alpha- and gamma-tocopherols used were the pure natural forms prepared by Dr. Meng in the Distillation Products Laboratories.

The sesame and olive oils used in some of the experiments were fresh samples of U.S.P. Grade. These were fed by droppers calibrated so that one drop was equivalent to 25 mg of the oils.

*Essential fat acid deficiency in rats*

Male albino weanling rats of the Sprague-Dawley strain were used. These were housed individually in an air-conditioned room maintained at 76°C. and 50% humidity. The rats were placed upon diet 60 at 40 to 45 gm body weight and maintained on this diet throughout the depletion period. When symptoms of the essential fat acid deficiency were evident the rats were divided into groups and started on curative experiments.

The criteria followed in these experiments were body weight, water consumption, severity of hind paw scaliness, and severity of caudal lesions. A discussion of these symptoms with respect to their use in assay procedures, and references to the original articles, has been given in a review by Burr ('42). Weekly records were kept of the symptoms.

Water consumption records were obtained by using covered cups with a 1-inch opening, wired to the side of the cage to prevent spillage. Weights of the cups plus water were taken at the beginning and end of a 24-hour period. A correction for water loss due to evaporation was obtained by use of identical cups in empty cages. This correction amounted to 3.0 to 3.5 gm. Usually the water consumption was determined on two adjacent 24-hour periods and the results averaged to give the daily water consumption for that week.

The severity of hind paw scale and caudal lesions was determined by weekly inspection of the rats. A numerical rating system, with 9 degrees, was used, ranging from "0" for freedom from symptoms, to "4" for maximum lesions, proceeding in " $\frac{1}{2}$ " steps. In hind paw scaliness, the first stage (" $\frac{1}{2}$ ") was denoted by the first appearance of scale between the toes. The next major division was at the scale rating "2" when the scale had covered the top of the foot

and had appeared around the ankle. Stage "3" involved the first appearance of a slight dermatitis around the toes, and stage "4" represented this condition aggravated still further.

In judging caudal lesions the " $\frac{1}{2}$ " stage indicated slight swelling and reddening of the tip (1 cm) of the tail, accompanied by slight circular ridging and the presence of a "fish scale" appearance for 1 or 2 cm above this. The condition progressed both in degree of the tail involved and in the severity of the lesions. Stage "2" involved the "fish scale" appearance over  $\frac{2}{3}$  the length of the tail, usually with necrosis setting in at the tip, and circular ridging fairly marked above this. Stage "3" was indicated by definite necrosis of the final inch of tail and marked progression of the circular ridging for  $\frac{2}{3}$  the length. In stage "4" the necrotic end had usually dropped off; the ridging had become acute with multiple open lesions and occasional bleeding. The tail was somewhat swollen, hard, and completely lacking in flexibility. The administration of curative agents reversed these steps.

The weekly numerical value for the lesion ratings of each group of rats on experiment was obtained by averaging the ratings of the individual members of the group.

In evaluating the results obtained it is felt that much more reliance can be placed upon growth and water consumption records, since these are obtained by physical measurements, than on lesion ratings which involve personal judgment. However, the ratings of the lesions do offer valuable corroborative evidence.

*Rate of onset of fat acid deficiency as  
influenced by tocopherol*

Sixty-two weanling rats were placed on diet 60. Eight were given 1 mg alpha-tocopherol daily, three were given 0.1 gm olive oil, equivalent to 6 mg linolate, and three were given both the olive oil and tocopherol supplements, daily. The remaining 48 were kept as controls. The average rates of change in body weights, water consumption, hind paw scale and caudal lesions are shown in figure 1.

Alpha-tocopherol, alone, had a slight beneficial effect on growth and delayed the onset of the typical lesions. This was especially true of the caudal lesions; up to 6 weeks on the depletion diet 50% of the rats receiving the tocopherol supplement had a caudal lesion rating of " $\frac{1}{2}$ ", the rest being still entirely normal. However, at this time 27% of the

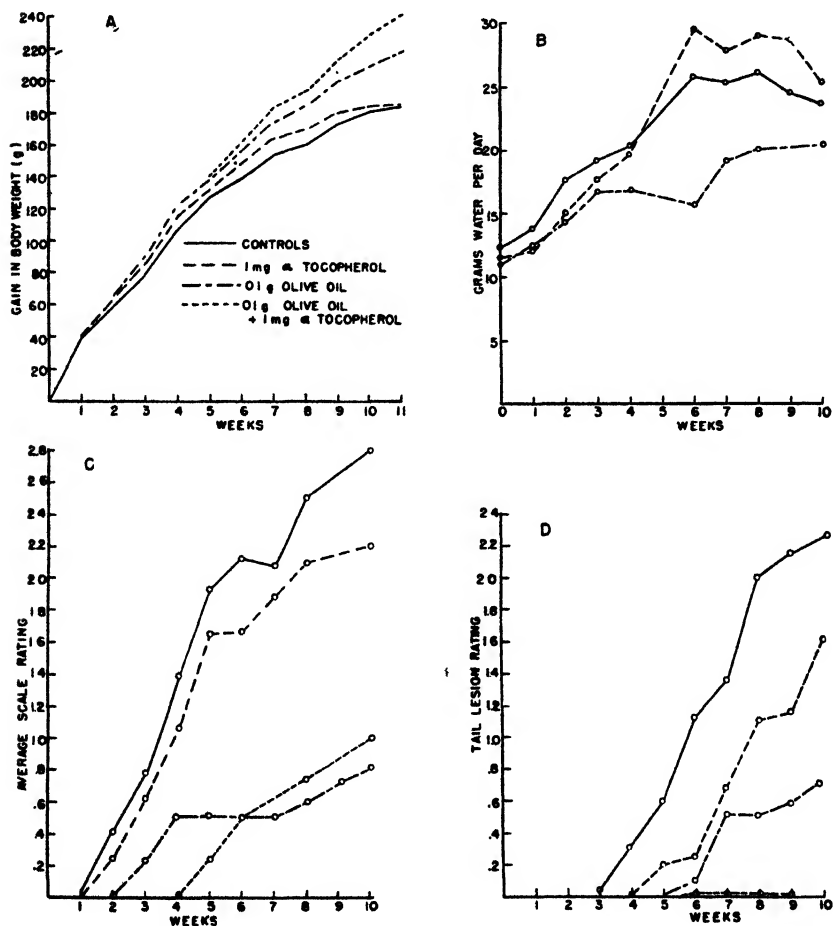


Fig. 1 Rate of depletion of essential unsaturated fat acid as influenced by tocopherol. The indicated supplements were fed daily. (A) Gain in body weight; (B) Average daily water consumption; (C) Average estimation of degree of scale on hind paw; (D) Average estimation of degree of caudal lesions.

control rats had severe tail lesions with ratings between "2" and "4" while only 19% were still normal. After 6 weeks on the depletion diet the rats receiving tocopherol rapidly began to develop the typical fat acid deficiency symptoms. This was especially noticeable in the water consumption records; the tocopherol-fed rats reached and maintained a higher water consumption level than the negative controls.

Alpha-tocopherol had a more marked beneficial effect on the growth rate and lesion prevention when given in addition to 6 mg of linolate (as olive oil). This level of linolate, alone, delayed, but was insufficient to prevent, the onset of the deficiency symptoms.

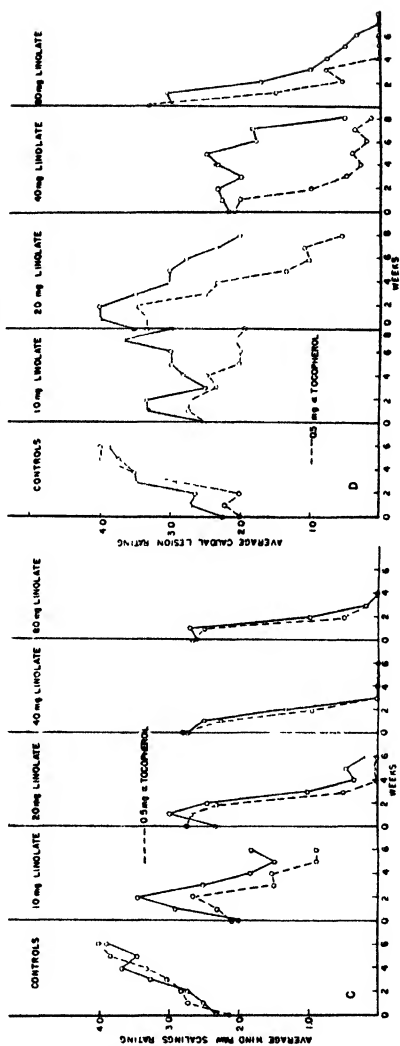
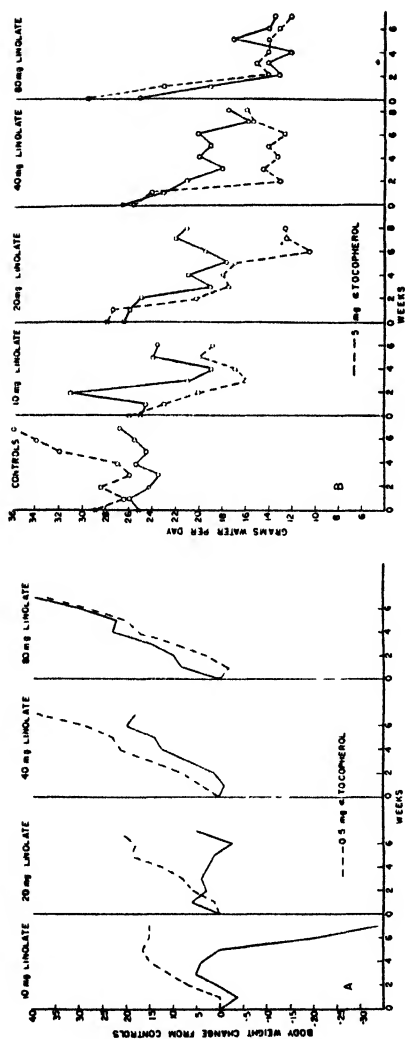
Similar results were obtained in another rate-of-depletion experiment. After 9 weeks on the depletion diet the average increases in body weight for groups of six rats receiving daily no supplement, 0.5 mg alpha-tocopherol, 10 mg linolate (as sesame oil), and the tocopherol plus the linolate, were 118, 127, 130, and 144 gm, respectively. The average tail lesion ratings at this time were 1.1, 0.5, 1.4, and 0.2, respectively.

*Curative action of methyl linolate as influenced  
by alpha-tocopherol*

Thirty rats with uniform deficiency symptoms were selected from the 48 controls of the previous experiment after they had been on diet 60 for 9 weeks. They were divided into 10 groups of three each. Five groups received supplements of methyl linolate six times weekly at levels of 0, 10, 20, 40, and 80 mg. The other five groups received the same methyl linolate supplements but with 0.5 mg of alpha-tocopherol in addition.

Figure 2 shows graphically the results of this curative experiment. The growth records have been expressed in

Fig. 2 Effect of tocopherol on the curative action of methyl linolate fed to essential fat acid deficient rats. The broken lines represent responses of rats fed 0.5 mg  $\alpha$ -tocopherol daily, in addition to indicated linolate supplements.



terms of the body weight change of the supplemented animals as compared with that of the negative controls. The other criteria are expressed in terms of their absolute values. The control animals gained an average of 54 gm during the 8-week curative test.

Alpha-tocopherol improved the growth response to low levels of linolate, but at the highest level (80 mg) of linolate was of no additional benefit. Similarly, the alpha-tocopherol supplements increased the effectiveness of the low levels of linolate as measured by the rate of decrease of water consumption, or by the rate of cure of the caudal lesions. The rate of cure of the hind paw scaliness shows little if any benefit from the tocopherol. However, even without tocopherol, this symptom was cured very rapidly, and showed no grading of response with variation in the linolate level.

Alpha-tocopherol, alone, did not influence growth, but did bring about a noteworthy increase in water consumption. This is similar to the results shown in figure 1B.

*Curative action of sesame oil as influenced  
by alpha-tocopherol*

There appears to be a rough proportionality between the linoleic acid and tocopherol content of most fats and oils. However, sesame oil is an exception. It contains about 40% linoleic acid and less than 5  $\mu$ g of tocopherol per gm. Therefore, sesame oil should be a good source of linolate for testing the interrelation of tocopherol and an essential fat acid in a triglyceride combination.

Thirty rats were depleted for 5 weeks on diet 60. Daily supplements of 0, 25, and 100 mg of sesame oil, with and without 0.5 mg of alpha-tocopherol, were started at this time. The group receiving each supplement was composed of six animals, except for the higher level of sesame oil, with and

without tocopherol. There were three animals in each of these two groups.

The influence of tocopherol on the curative action of sesame oil is shown in figure 3. The effectiveness of the lower level of sesame oil was clearly increased by the tocopherol sup-

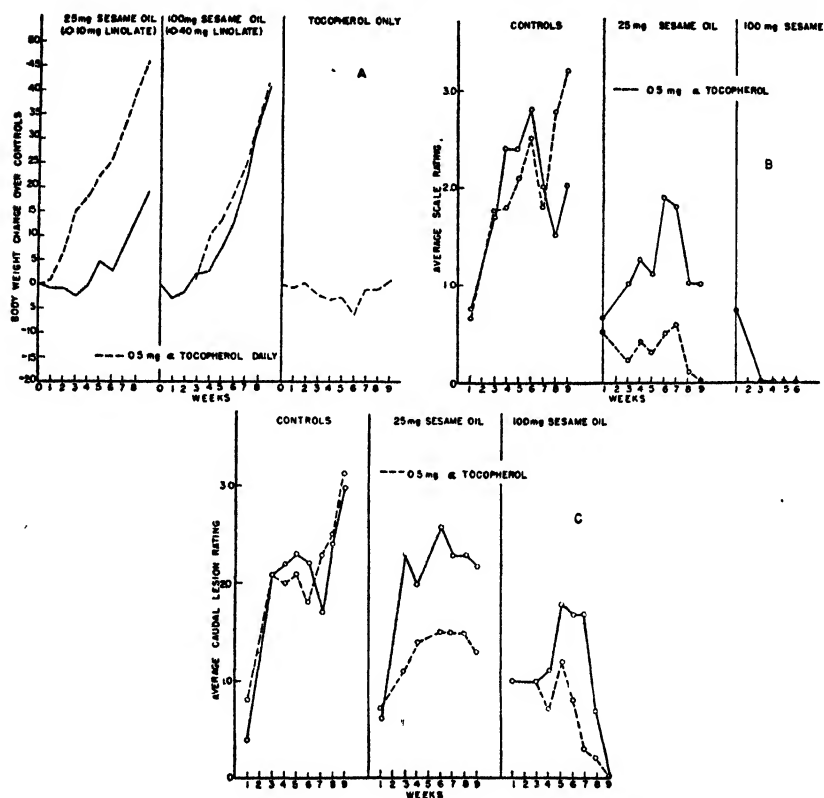


Fig.3 Effect of tocopherol on the curative action of sesame oil fed to fat acid deficient rats. The broken lines represent responses of rats fed 0.5mg  $\alpha$ -tocopherol daily, in addition to the indicated sesame oil supplements.

plement, as is indicated by the growth, hind paw lesion, and caudal lesion charts. At the higher level of sesame oil this beneficial effect of tocopherol is not so evident. The control rats receiving no supplement gained an average of 79 gm during the 9 weeks' curative experiment.



Alpha-tocopherol, alone, had little influence on growth, but the rats in this group developed ear and nose lesions and a general unthrifty appearance not evident in the negative control group.

*Variation of the level of alpha-tocopherol*

Twenty-four rats which had been on diet 60 for 12 weeks since weaning were separated into six groups. One group served as the negative control, another received 0.5 mg of alpha-tocopherol per rat, daily. The rats of the four remaining groups were given 10 mg of methyl linolate daily, and in

TABLE 1

*Response of fat acid deficient rats to suboptimal doses of methyl linolate plus various levels of  $\alpha$ , alpha-tocopherol.*

(Four rats per group. Supplements started after 12 weeks, on depletion diet 60.)

DAILY SUPPLEMENT	WEIGHT CHANGE IN 6 WEEKS	WATER INTAKE CHANGE IN 6 WEEKS	HIND PAW LESION CHANGE IN 6 WEEKS
	<i>gm.</i>	<i>gm/day</i>	
None	17.7	+ 0.7	
0.5 mg $\alpha$ -tocopherol	14.0	+ 2.5	
10 mg Me-linolate	21.5	- 1.3	- 0.8
10 mg linolate + 0.25 mg $\alpha$ -tocopherol	55.0	- 7.2	- 1.2
10 mg linolate + 0.5 mg $\alpha$ -tocopherol	35.0	- 8.6	- 1.6
10 mg linolate + 1.0 mg $\alpha$ -tocopherol	52.3	- 11.0	- 1.0

addition received 0, 0.25, 0.5, or 1.0 mg of alpha-tocopherol daily. These supplements were continued for 6 weeks. Their effect on the average change in body weight, and the average change in daily water consumption over this period, is given in table 1.

The group receiving linolate alone gained an average of 3.8 gm more than the negative controls, while the average gain of the animals receiving linolate and 0.25 mg of tocopherol was 37.3 gm more. Larger amounts of tocopherol did not improve the growth performance. However, water consumption declined steadily as the tocopherol was increased to 1 mg daily.

*Alpha- vs. gamma-tocopherol as sparing agents*

It has been shown by Hickman, Kaley and Harris ('44) that gamma-tocopherol is equivalent to the alpha form as a sparing agent for vitamin A, *in vivo*. The relative potencies of these two compounds as sparing agents for methyl linolate

TABLE 2

*The protection of suboptimal levels of methyl linolate in curing the fat acid deficiency.*

(Six rats per group. Supplements started after 6 weeks — A, and 7 weeks — B, on diet 60.)

DAILY SUPPLEMENT	AVERAGE STARTING BODY WEIGHT		AVERAGE BODY WEIGHT INCREASE IN 5 WEEKS	
	gm	Mean deviation	gm	Standard error
A. Comparison of $\alpha$ - and $\gamma$ -tocopherol				
None	156.5	$\pm 19.2$	30.7	$\pm 2.3$
0.5 mg $\alpha$ -tocopherol	169.6	$\pm 10.5$	28.3	$\pm 1.9$
20 mg linolate	154.9	$\pm 7.7$	50.7	$\pm 2.6$
20 mg linolate plus 0.5 mg $\alpha$ -tocopherol	159.8	$\pm 14.0$	65.7	$\pm 3.2$
20 mg linolate plus 0.5 mg $\gamma$ -tocopherol	155.0	$\pm 6.5$	58.5	$\pm 1.8$
B. Effect of feeding on alternate days				
None	194.3	$\pm 15.0$	25.4	$\pm 3.5$
20 mg linolate daily	173.5	$\pm 20.5$	43.8	$\pm 3.5$
40 mg linolate fed every other day	183.8	$\pm 9.5$	45.5	$\pm 3.4$
20 mg linolate plus 0.5 mg $\alpha$ -tocopherol daily	186.0	$\pm 9.7$	51.8	$\pm 2.3$
40 mg linolate plus 0.5 mg $\alpha$ -tocopherol fed on alternate days	202.0	$\pm 6.7$	56.0	$\pm 1.9$

were tested. The results given in table 2-A indicate slightly less activity for the gamma-tocopherol. However, on statistical analysis the difference in response to the two tocopherols falls somewhat short of significance by the *t* test ( $P = 0.05$ ).

The animals were depleted for 6 weeks on diet 60, after which supplements were begun, using six rats per group. After a 5-week supplementation period the group receiving 20 mg of linolate, alone, showed an average gain of 20.0 gm more than the controls. When alpha-tocopherol was given in addition, the growth gain averaged 35.0 gm. With gamma-tocopherol plus the linolate the growth gain averaged 27.8 gm.

### *Separate feeding of linolate and tocopherol*

After 7 weeks' depletion on diet 60, rats were placed upon five supplemental variations using six animals per group. One group served as the negative control. Two other groups received, daily, 20 mg of methyl linolate; one of these was given 0.5 mg of alpha-tocopherol daily, in addition. The two remaining groups were given, every other day, 40 mg of methyl linolate; one of these groups received 1 mg of alpha-tocopherol on the days alternating with the linolate feeding. The experiment was continued for 5 weeks. The effect of these supplements on the growth of the animals is shown in table 2-B.

The average growth of the two groups receiving linolate was about the same. Alpha-tocopherol gave practically equivalent benefit whether fed daily with the linolate or on alternative days.

### DISCUSSION

The data indicate that alpha-tocopherol extends the effectiveness of suboptimal quantities of linolate in preventing or curing the essential fat acid deficiency in rats. Gamma-tocopherol is also an effective sparing agent for low levels of linolate, but possibly less so than the alpha form.

The sparing action appears not to be limited to the gastrointestinal tract, since the onset of the deficiency syndrome was delayed by tocopherol given during the depletion period. Furthermore, it exerted a sparing action on low curative levels of linolate when fed separately at 24-hour intervals. This indicates a sparing of linolate in liver or tissue cells or

in body fluids, unless it is assumed that the tocopherol remained in the stomach for as long as 1 day.

When the deficient animal is flooded with large doses of linolate alone, rapid and complete cure results. Tocopherol fed in addition to these doses exerts no evident benefit on the rates of cure. Since additional tocopherol is not essential to the complete cure, the observed sparing of low levels of linolate must be due to a protective action, and is not simply the response to two independent deficiencies. It does not follow, necessarily, that because tocopherol gives no apparent benefit with sufficiently large doses of linolate, no interrelation exists between vitamin E and linolate in the ultimate biochemical function of the latter. Body tissue cells probably still contain fairly high levels of vitamin E even though the stores in liver and blood, needed for the *in vivo* sparing action, may be low.

Alpha-tocopherol given without linolate to rats which are suffering from an essential fat acid deficiency aggravates the symptoms. Two possible explanations are, either that the tocopherol acts as a prooxidant under these conditions and accelerates the destruction of the residual linolate, or that the tocopherol, acting as a physiological antioxidant blocks out more completely the normal and necessary oxidation of the residual linolate. The latter explanation implies that peroxidation at the central double bonds is involved in the essential biochemical function of linolate.

No proof exists for such peroxidation, *in vivo*, and there is evidence that complete central cleavage of the carbon chain does not occur. However, peroxidation may serve a function without leading to complete cleavage. Linolate peroxides may act as a potent terminal hydrogen acceptor for some chain of enzymatic oxidation, such as the  $\beta$ -oxidation of fats.

Salmon ('38) has noted that pyridoxine exerts a sparing action on essential fat acid. The structure of pyridoxine indicates that it should have antioxidant activity. To test this, freshly prepared free base pyridoxine was added at 0.08% to a vitamin A solution in fish oils. Incubation at 55°C.

showed that the stability of the vitamin A was doubled. This antioxidant activity of pyridoxine may be involved in the observed sparing of linolate, *in vivo*.

The significance to human nutrition of the sparing of linolate by tocopherol is complicated by lack of agreement concerning the essential nature of the unsaturated fat acids. There is evidence that infantile eczema is related to a deficiency in essential fat acids (Brown and Hansen, '37). The daily human requirement for linolate may be tentatively placed at 3 gm by calculating, on the caloric basis, from the rat's requirement of about 1 mg per calorie. Three grams of linolate would be contained in about one ounce of lard, or a quarter of an ounce of some vegetable oils. For efficient utilization of this quantity of linolate about 30 mg of tocopherol daily may be necessary. This calculation is based on the apparent ratio of 100 to 1 shown to give good protection in the rat.

#### SUMMARY

Alpha-tocopherol extends the effectiveness of suboptimal quantities of linolate in preventing or curing the essential fat acid deficiency syndrome in the rat. This sparing action has been shown using pure methyl linolate or sesame oil. Gamma-tocopherol also spares essential fat acid, *in vivo*.

The interrelation of tocopherol and linolate appears not to be restricted to the gastrointestinal tract, since feeding of these substances separately at 24-hour intervals still shows enhanced growth, as compared with either supplement by itself.

When tocopherol but no essential fat is fed to fat-deficient rats the deficiency symptoms are aggravated.

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# BIOLOGICAL VALUE OF PROTEINS IN RELATION TO THE ESSENTIAL AMINO ACIDS WHICH THEY CONTAIN<sup>1</sup>

## III. COMPARISON OF PROTEINS WITH MIXTURES OF THE AMINO ACIDS

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That the essential amino acids must be determining of the biological value of proteins is so obvious as scarcely to require any introductory comment (Thomas, '30; Rose, '38). It is important now to learn (1) whether a mixture of the ten essentials in the proportion one to another contained in a protein can produce as much retention of nitrogen in relation to the amount absorbed, as can be obtained from the same amount of total nitrogen supplied by the protein itself, and (2) to what extent allowance must be made for the unnatural isomers of the racemic (*dl*) forms which cannot be utilized.

Before entirely artificial proteins are adopted for human nutrition—even emergency nutrition—it should be borne in mind that no proof has ever been given that all protein in the alimentary tract is broken down to the stage of biuret-free products before absorption (Van Slyke, '42). This being true it is possible that certain preformed aggregates (poly-

<sup>1</sup> The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Rochester.



peptids) are advantageous to protein nutrition. It is certain that entirely separate amino acids ingested in solution are absorbed more rapidly than natural products formed by proteolytic alimentary enzymes, and that a measurable portion escapes deamination, being consequently excreted unchanged in the urine. The question from this standpoint, therefore, is one of economy of utilization.

The supreme test of adequacy of a mixture of essential amino acids representing a protein as compared with the protein itself would be to feed the protein first, following a no-protein period, and immediately afterward a mixture of the ten in the same proportion one to another in which they occur in the protein and in sufficient quantity to supply the same total nitrogen as the protein. The amounts of the essentials over and above that represented by them in the protein should be sufficient to form all the nonessential amino acids supplied by the protein for whatever measure of usefulness they possess for the body. This was the hypothesis upon which the comparisons reported in this paper were based.

Obviously it was necessary first to learn the composition of the proteins selected with respect to the ten essentials. This analytical work will be reported in detail in a separate publication,<sup>2</sup> only such parts of it being used in this report as are necessary for clarity of presentation.

#### PROCEDURE

When this project was undertaken (August, 1943) only the racemic forms were available of isoleucine, leucine, methionine, phenylalanine, threonine, tryptophane and valine. For the basic amino acids arginine, histidine and lysine the *l* (+) monohydrochlorides were most available. All these<sup>3</sup> were

<sup>2</sup> A grant was obtained from the Nutrition Foundation and Dr. R. R. Sealock, at that time assistant professor in the department, agreed to supervise the analytical work. After Dr. Sealock left (January 1945) to become associate professor of biochemistry at Ames, Iowa, the responsibility was taken over by one of us (L.E.E.) and, when teaching duties interposed, it was passed along to Dr. Grant Bartlett, who served until the end of the project.

<sup>3</sup> Purchased from Merck and Company, Rahway, New Jersey.

confirmed as to purity by Kjeldahl determination of the nitrogen contents and as to freedom from heavy metals by spectrographic analysis very kindly performed for us by Dr. L. T. Steadman of the radiology department.

The source material of the protein having been analyzed, and the desired level of feeding, gauged by the urinary nitrogen excretion of the second or third day of no protein, having been decided, the test protein could be started; but the level of intake often had to be adjusted after a day or two, when the "no-protein" fecal nitrogens became available. Only the last 2 or 3 days' urines of each period were used in the calculations. Because of unpredictable fecal losses from some of the food proteins these calculations involved appreciable error, but the aim was not to secure the most reliable biological values so much as to rate protein and amino acid mixture against the same basis of calculation. Paper I (Murlin et al., '46a) sets forth the issues which had to be dealt with here.

The analysis of a protein being in hand, the mixture of essential amino acids was compounded on the as-purchased basis to give as nearly as possible, taking into account anticipated larger urinary losses, a nitrogen balance at least as high as that of the protein. This mixture, fed in the period following the test protein, replaced it in the total diet (see paper I for the basal diet).

The amino acids were dissolved successively in distilled water in two or more 4-liter pyrex beakers over free flames, the temperature being kept below 80°C. to avoid discoloration of tryptophane. After 1 day's experience with each mixture the amount of water necessary could be estimated rather exactly. Pouring from one beaker to another was a decided help for obvious reasons, and as a rule the entire quantity daily for a squad of ten subjects could be dissolved in 6 or 7 liters at 80°C. The beakers then were partially cooled and their contents transferred, with liberal use of rinsing water to prevent crystallization, to 2-liter pyrex volumetric flasks and cooled to 20°C. The whole solution amounting to 8 or 10 liters was assembled in a 12-liter flask kept in the cooling water, and after thorough mixing each person's aliquot measured at 20° into bottles of appropriate size. Approximately one-third of the day's volume was consumed by sipping

frequently during each meal. The nitrogen content of the solution was checked by Kjeldahl analysis and almost always gave a result a little below the theoretical, even though the acids were kept in a desiccator.

The amino acid period varied from 3 to 6 days depending on how well the particular mixture was tolerated. If nausea was serious or vomiting occurred, the period was terminated at 3 days; if not it was continued to at least 4 days. Account always had to be taken of the remainder of the program for each series of experiments. If other "no-protein" and amino acid experiments were contemplated, it was not advisable to prolong the periods longer than 4 days because of the inevitable loss of weight during the amino acids regimes (see paper I, Murlin et al., '46a).

The subjects of these experiments were for the major part conscientious objectors assigned from their quota by O.S.R.D.

## RESULTS

Seven of the ten experiments with amino acid mixtures listed in table 1 were performed in the order and manner described under Procedure and give the pattern characteristic of the regular routine. Three of them departed from the pattern in one or more respects. The first in the table had its "no-protein" period following those for the protein and amino acids. The second corn germ experiment (6-iii and iv) made use of an amino acid mixture to bring 19.44 gm wheat germ protein to an equality with 22.29 gm corn germ protein. The second experiment with egg protein (6-vii and viii) employed glutamic acid and glycine to make up the nitrogen of egg not supplied by essential amino acids. These three therefore are to be regarded as atypical experiments in method but equally instructive with the others in results.

To consider these first, the low biological value of egg protein in series 3 is accounted for in part by the reverse order of the protein and no-protein periods and in part by the high level of protein intake resulting in a plus N balance (Allison and Anderson, '45). The depleting effect of a no-protein period certainly promotes retention of a protein consumed immediately afterward. The experiment with wheat germ plus amino acids in comparison with corn germ showed that

TABLE 1

*Synopsis of proteins and mixtures of essential amino acids consumed,  
nitrogen balances and biological values (B.V.).*

SERIES NO.	PERIOD NO.	CONSUMED	NO DAYS	NO. SUBJECTS	DAILY AVERAGES			B.V. of amino acids as % of protein B.V.
					Intake N	N bal	B V.	
3	viii	Whole egg	6	7	4.750	+ 0.374	75	
	ix	Amino acids	5	7	4.509	- 0.816	57	76
	x	No protein	3	7	0.333	- 2.853		
4	vi	No protein	3	11	0.292	- 3.411		
	vii	"Kitchen food yeast" <sup>1</sup>	5	11	3.700	- 1.003	83	
	viii	Amino acids	3	11	4.852	- 0.035	75	90
5	ii	No protein	3	10	0.361	- 3.481		
	iii	Cottonseed meal <sup>2</sup>	5	10	3.867	- 1.128	87	
	iv	Amino acids	3	10	4.353	- 0.599	74	85
	vii	No protein	3	7	0.324	- 3.038		
	viii	Corn germ	5	7	4.164	- 0.128	95	
	ix	Amino acids	5	7	4.247	- 0.123	83	87
6	ii	No protein	3	4	0.236	- 3.050		
	iii	Corn germ <sup>3</sup>	5	4	4.668	- 0.461	78.6	
	iv	Wheat germ <sup>3</sup> + Am. Ac. to equal corn germ	4	4	4.577	- 0.565	68.8	87
	vi	No protein	3	4	0.251	- 3.107		
	vii	Whole egg	5	4	3.305	- 0.184	101.7	
	viii	Amino acids, essential and nonessential	4	4	3.684	- 1.050	62.5	61
	x	No protein	3	2	0.433	- 3.029		
	xi	Haddock	5	2	3.245	- 0.685	92	
	xii	Amino acids	2	2	3.464	- 0.424	75	81
	ii	No protein	4	9	0.806	- 3.471		
	iii	Corn germ	5	9	4.394	- 1.061	96.5	
	iv	Amino acids	4	9	4.195	- 1.409	66	68
8	vii	No protein	4	9	0.526	- 2.769		
	ix	Corn germ	7	9	4.896	- 0.465	75.5	
	x	Amino acids adjusted	4	9	6.235	+ 0.023	54.7	74
	xii	No protein	4	8	0.195	- 2.842		
	xiii	Beefsteak	6	8	3.889	+ 0.484	91	
	xiv	Amino acids	3	8	3.983	- 0.473	67	74
		Amino acids adjusted	2	8	4.667	- 0.061	65	71

<sup>1</sup> Supplied by Anheuser-Busch Co. of St. Louis, Mo.<sup>2</sup> Obtained from Oil Mill Products Co., Ft. Worth, Tex.<sup>3</sup> Obtained from the VioBin Corp., Monticello, Ill.

by deducting the nitrogen of the unnatural isomers of the *dl* forms added to wheat germ the biological value of this protein could be brought into equality with that of corn germ (see footnote 9). The second experiment with egg makes it evident that neither glutamic acid nor glycine nor both aids retention of the total nitrogen represented by essentials and nonessentials.

It is clear from table 1 that none of the experiments with mixtures of essential amino acids compounded according to the analysis of the protein for these constituents, and supplying as much or more (three exceptions) nitrogen as the protein, produces a biological value so high as the protein itself when consumed by the same persons and evaluated against the same endogenous nitrogen. Expressed as a percentage of the B.V. of the protein, the B.V. of the corresponding mixture varied from 61% (series 6, period viii, essentials and nonessentials) to 90% (4-viii, amino acids containing nitrogen enough to approach equilibrium closely). There is no regular pattern of performance on the amino acid diet as compared with that on protein. This was scarcely to be expected.

The failure of such mixtures to duplicate the biological values of the proteins, which they imitated with respect to these ten constituents more or less exactly, was not surprising in view of the facts developed by Rose and co-workers (Rose, '38), Totter and Berg ('39), Berg ('42), Bauer and Berg ('43), Albanese ('44) and others regarding the nutritional inequality of the *d* and *l* isomers. According to the reviews by Rose and Berg the unnatural isomers of isoleucine, leucine, valine and threonine are not utilized at all for maintenance and growth in rats, and according to Bauer and Berg they are also not available to the mouse. Totter and Berg ('39) reported that the unnatural isomers of tryptophane and histidine were not so effective in the mouse as they have been found to be for the rat, and that unnatural lysine is not effective at all in either species. While Bauer and Berg found both optical isomers of phenylalanine and methionine apparently equally well utilized for growth in mice (as they are in

the rat), Albanese believes on the basis of his excretion studies that the unnatural isomers of tryptophane and phenylalanine are not readily utilized (retained) by man. Since differences have been found between two species so closely related as the rat and mouse with respect to the utilization of unnatural tryptophane, histidine and possibly leucine, Albanese expresses the view that "it is obviously unsafe to make assumptions about man on the basis of animal studies."<sup>4</sup> However, accepting the results on rats and mice as at least indicative of what may be expected in maintenance metabolism in man, and the scanty evidence obtained directly from human subjects, it appears that there are six *dl* forms, isoleucine, leucine, phenylalanine, threonine, tryptophane and valine, whose unnatural isomers may be rejected by man. Hence it does not appear at all gratuitous to assume that under the conditions of these experiments when sufficient amounts of the natural isomers are supplied the unnatural ones should be regarded as completely dispensable.

The problem then was, first, to determine whether an overall correction could be made in the mixture to compensate for this inequality. If it were certain that all of the nitrogen of the unnatural form in the *dl* isomers were wasted without being deaminated, it would be justifiable to deduct the total amount of this nitrogen from the absorbed and urinary nitrogens and recalculate the biological value on the remainder absorbed and excreted. This is regularly done in this laboratory for the purine nitrogen of coffee, tea and carbonated beverages, because they are largely excreted unchanged in the urine. The same treatment would be proper for medicinal nitrogen or for that of urea added to the diet as a test substance for kidney function. Rose ('38, p. 131) states it is customary in his laboratory and elsewhere "if a synthetic product is employed to double the amount in order to insure the presence of the active isomer at the desired level." This procedure assumes that none of the unnatural isomer is util-

<sup>4</sup>One is reminded of Mitchell's ('38) comment: "It is good that our complacency in the infallibility of a sort of Jeffersonian doctrine that all animals are created equal is being disturbed."

ized for retention and has been tried several times in this study, but with no better results, as will be seen in table 2. Without correction the B.V. is too low; with correction for all the nitrogen of the unnatural isomers it is too high.

All three of the derived values in the examples given overcorrect the B.V. of the proteins. Likewise all the other experiments in table 1 revealed overcorrection when all the nitrogen of the unnatural forms was deducted. This obviously means that some of it, but a varying proportion from experiment to experiment, was utilized in retention, therefore presumably in true anabolic processes. From present knowledge

TABLE 2

*Theoretical correction for N of unnatural isomers in experiments with egg and yeast proteins.*  
(All values are average for entire squad.)

	a	b	c	d	e	f	g	h	i	j	k
	Test protein fecal N	No-protein fecal N	(a-b) Fecal waste N	Test protein N eaten	(d-e) Absorbed N	True digestibility %	Test protein urine N	No-protein urine N	(g-h) urinary waste N	i × 100 e	(100-j) B.V.
<b>Egg</b>											
(3-viii)	0.970	0.822	0.148	4.750	4.602	97	3.406	2.350	1.156	25.0	75
Am. Ac.											
(3-ix)	1.192	0.822	0.370	4.510	4.140	92	4.133	2.350	1.783	43.0	57
					1.289		1.289				
Correction for all N of unnatural isomers:					2.851		2.844	2.350	0.494	17.3	82.7 <sup>1</sup>
<b>Yeast</b>											
(4-vii)	1.544	1.074	0.470	3.700	3.230	86	3.159	2.629	0.530	16.4	83.6
Am. Ac.											
(4-viii)	0.773	1.074	— 0.301	3.772	4.073	108	3.653	2.629	1.024	25.1	74.9
					1.046		1.046				
Correction for all N of unnatural isomers:					3.027		2.607	2.629	— 0.022	0.07	100.1 <sup>1</sup>
Am. Ac.											
(4-viii)	0.773	1.074	— 0.301	4.852	5.153	94	4.116	2.629	1.487	28.9	71.1
doubling dl forms					1.365		1.365				
Correction for all N of unnatural isomers:					3.788		2.751	2.629	0.122	3.5	96.5 <sup>1</sup>

<sup>1</sup> Overcorrected.

it is not possible to deduce from the literature a single correction factor to compensate exactly for the nutritional inequality in the case of each of the seven racemic forms used in this study.

The literature does, however, clearly reveal that the unnatural isomers are the ones, which, though they may be made to fit the body needs in emergency perhaps after acetylation (duVigneaud, Sealock and Van Etten, '32; Holt and Albanese, '45), either like foreign or medicinal substances or like nonessential surplus amino acids, by oxidative deamination. If any unnatural isomer should be, like ingested urea, wholly useless, it would be found in the amino acid fraction of the urinary nitrogen. If like nonessential amino acids it should be deaminated and transformed into urea or ammonia it would be found in one or the other of these fractions. Can we not then change the level of feeding so that, assuming the same rate of performance of the human organs, the whole of the nitrogen of these scarcely assimilable intruders would be rejected in one form or another?

It is not difficult to calculate how much nitrogen would need to be subtracted from the absorbed and urinary nitrogens in any experiment with the amino acids to give the correct biological value. The formula is the following:

$$(1) \quad \text{B.V. of the Protein as percentage} = \frac{\left[ \frac{\text{Retained N}}{\text{Absorbed N-x}} - (\text{Ur.N-x}) - \text{No-Prot.Ur.N} \right] \times 100}{\text{Absorbed N-x}}$$

x will be something less than the total nitrogen of the unnatural isomers fed. Suppose it were two-thirds of the total. This would imply that one-third of the nitrogen of the unnatural forms already is being excreted. If so, increase of the *dl* isomers by two-thirds should permit excretion of the total nitrogen of these forms as "medicinal" or useless nitrogen, because then the nitrogen of the natural forms would be sufficient, having also been increased by two-thirds.

An experiment performed late in the course of the investigation illustrates just such a correction. At the time the



analyses of the hydrolysates for the essential amino acids were more satisfactory, because confirmed by high percentage recoveries of pure amino acids added singly to the hydrolysates. Likewise the fourth-day no-protein urinary nitrogen excretions were dependable as endogenous base according to the criteria developed in paper I (Murlin et al., '46a). The experiment referred to will be found in series 8, periods ix and x, of table 1.

*Corn germ protein and its amino acid counterpart*

The series started with a new squad of nine men. Corn germ was again chosen as source of the test protein. A preliminary experiment on this protein followed by the corresponding mixture of the ten essential amino acids had been run for the express purpose of deriving a correction factor for the nitrogen of all the unnatural isomers taken together, which could be applied in a repetition of the experiment. Urinary nitrogens of the first no-protein period (8-ii) were not yet low enough for a dependable absolute biological value; but it was decided, since the men were scheduled for at least two more such periods, to accept a high B.V., because whatever its level a correction derived by comparison of protein and amino acid mixture referred to the same base probably should apply satisfactorily later against true endogenous excretions.

The calculation by means of equation (1) above produced a value for  $x$  very close to two-thirds (66.9%)<sup>5</sup> of the total nitrogen of the unnatural isomers fed and, as it happened, just 33.3% of the total average amino acid nitrogen fed in the preliminary experiment; namely, 3.977 gm daily. The nitrogen balances were too low as may be seen in table 1 (8-iii and iv), to protect body protein. It was desirable from this point of view also to increase the nitrogen intake. Allison and Anderson ('45) have shown in careful experiments on dogs that B.V. is a constant so long as absorbed nitrogen and nitrogen balance from the same protein bear a linear relation-

<sup>5</sup> On recalculation an error was found, correction of which made the percentage 60.3%, but it was then too late to change.

ship to one another, but this applies only to the region of negative balances. Their values are expressed in terms of surface area. But whatever the mode of expression it is evident that where there is excessive excretion, as in these experiments, due to the more rapid absorption of amino acids than of products of protein digestion and to the relatively lower nutritional value of at least most of the unnatural isomers, if not all of them, no such relationship could be expected, between protein and amino acid mixture.

The corn germ feeding was increased by 0.75 gm nitrogen (average per man) and produced a B.V. of 75.5 (table 3) (Mitchell and Beadles, '44). Increasing the *dl* amino acids by 66.9% above the level calculated to equal the nitrogen intake on corn germ and maintaining the percentages one to another of these seven constituents produced the B.V. of 54.7. The three basic amino acids were held at the calculated level before increasing the *dl* forms, and as a consequence the total nitrogen of the unnatural isomers now amounted to 28% of the total nitrogen of all the amino acids, 5.982 gm, instead of 33.3% for the preliminary experiment. Applying

TABLE 3

*Biological value of corn germ protein (period 8-ix, table 1).*

c	d	e	f	g	h	i	j	k	
Fecal N correction	Test protein N fed	(d-e) absorbed N	True digest- ibility	Test protein urine N	No-protein urine N	(g-h)	$\frac{i \times 100}{e}$	100-j B.V.	N-bal.
1.147	4.896	3.749	77	3.133	2.215	0.918	24.5	75.5	- 0.465
Biological value of amino acid mixture with <i>dl</i> forms increased by 66.9% (period 8-x, table 1).									
0.166	6.235	6.069	97	4.966	2.215	2.751	45.3	54.7	+ 0.023
Correction for all N of unnatural isomers									
fed:		1.675		1.675					
		4.394		3.291	2.215	1.076	24.5	75.5	+ 0.023
Correction for excess of urea, NH <sub>3</sub> and Am.									
acid N in urine:		6.069		4.966					
		1.712		1.712					
		4.357		3.254	2.215	1.039	23.8	74.2	+ 0.023

this correction on the assumption that all of this nitrogen of the unnatural isomers is now dispensable, the B.V. of the protein is reproduced precisely.

This experiment falls short of perfection, however, for the proper correction factor was not used (see footnote 5). But if the factor 60.3% had been used, the effect on B.V. would have been very small.<sup>6</sup> A further check on the correction for a fraction of the total nitrogen equal to that of the unnatural isomers, is found by taking into account the distribution of nitrogen in the urine, as will be explained more fully in connection with the beefsteak experiment. The extra urea and ammonia N deriving from the adjusted amino acid intake was estimated at 1.299 gm and the extra amino acid nitrogen equal to 0.483 gm, a total of 1.712 gm. Deducting 1.712 gm, in place of the 1.675 gm representing the total nitrogen of the unnatural isomers, produces a B.V. of 74.2 (table 3). There is of course no way of proving that all the extra urea, ammonia and amino acid nitrogen<sup>7</sup> came from the unnatural isomers, but it is at least a striking coincidence that the sum of these fractions so nearly equalled just one-half of the total nitrogen of the *dl* isomers at the adjusted level.

Nitrogen balances of protein and its amino acid counterpart need not be equal to give equal biological values (table 3); but it is important that the nitrogens absorbed be at least close together and the correction accomplishes this with reference to the isomers of the amino acids known to be available for synthesis.

This demonstration creates a strong probability, though not direct proof, that when sufficient amounts of natural isomers are present the unnatural forms become entirely dispensable.

<sup>6</sup> Thus the total N of the unnatural isomers would have been 27.5% of the total amino acid nitrogen, the correction 1.604 gm instead of the 1.675 gm used in table 3, and the B.V. 73.5. This assumes that the urinary excretion had remained the same. It could not have been higher, and if it had been reduced, the limit would have been 0.132 gm; consequently the B.V. would have fallen between 73.5 and 76.7.

<sup>7</sup> A few tests performed by Dr. Grant Bartlett, employing a *d*-amino acid oxidase, prepared from acetone powders of rabbit kidney and liver, estimated the *d*-amino acid nitrogen on 2 days of this period at 17 to 21% of the total amino acid nitrogen in the urine or about 10.4% of the nitrogen of the unnatural isomers fed.

Such a result could not have been obtained if the excretion data had not been entirely reliable.

Another and perhaps more pertinent criticism would be that by increasing the *dl* forms alone, leaving the basic amino acids unchanged, the proportions of the latter necessarily were reduced and the former increased to such an extent that the resulting mixture no longer duplicated the composition of the corn germ protein. A look at the two mixtures before and after adjustment of the *dl* forms shows how much they were changed (table 4).

TABLE 4

*Distribution of the essential amino acids in corn germ protein and in the mixture of synthetic amino acids (on the as-purchased basis, containing 66.9% increase of dl forms).*

CORN GERM PROTEIN <sup>1</sup>		AMINO ACID MIXTURE <sup>2</sup>	
	%		%
Arginine	16.19	<i>l</i> (+) Arginine	12.88
Histidine	5.87	<i>l</i> (+) Histidine	5.17
Isoleucine	6.48	<i>dl</i> Isoleucine	7.04
Leucine	18.94	<i>dl</i> Leucine	20.57
Lysine	12.28	<i>l</i> (+) Lysine	10.99
Methionine	7.83	<i>dl</i> Methionine	8.51
Phenylalanine	9.52	<i>dl</i> Phenylalanine	10.34
Threonine	9.16	<i>dl</i> Threonine	9.95
Tryptophane	6.11	<i>dl</i> Tryptophane	6.46
Valine	7.44	<i>dl</i> Valine	8.09
	99.33		100.00

<sup>1</sup> The essential amino acids make up 62.19% of the total protein ( $N \times 5.7$ ) of corn germ. The percentages given reveal the proportion of each to all and to one another in this 62.19%.

<sup>2</sup> This is the adjusted or final mixture.

Comparison of the figures in table 4 makes evident that all the basic amino acids are reduced more than the *dl* forms are increased, the relative change of the former being from 10.5% for lysine to 19.8% for arginine; for the latter from 8.4% for leucine to 8.8% for valine.

Calculated on the percentage of the nitrogen of the *l* or natural forms, which is more important from the point of view of our working hypothesis, the change, as seen in table 5 (cols. 3 and 5) is found to be reversed. All of the basic amino acids

now are increased because all of their nitrogen is of the natural form, while all the nitrogens of the *l* isomers are lower than in the protein, because obviously only half of the nitrogen of the *dl* isomers is considered. The relative change is now 8.4% for lysine, 8% for histidine and 5% for arginine. The relative decrease among the *dl* forms runs from 9 to 11%. On the evidence in table 3 it is believed these alterations are not significant for biological values.

TABLE 5

*Distribution of nitrogen of the natural isomers among the essential amino acids of corn germ protein and the mixture of synthetic amino acids.*

	CORN GERM PROTEIN			AMINO ACID MIXTURE		
	Gm N/ 100 gm	Per cent of total N	Av. N Gm/man/day essentials only	Gm N/ 100 gm	Per cent of total N	Av. N Gm/man/ day
Arginine	3.27	32.3	0.932	3.27	34.9	1.502
Histidine	0.99	9.8	0.292	0.99	10.6	0.456
Isoleucine	0.43	4.2	0.121	0.36	3.8	0.166
Leucine	1.26	12.5	0.361	1.05	11.2	0.483
Lysine	1.46	14.4	0.418	1.46	15.6	0.674
Methionine	0.46	4.5	0.130	0.38	4.0	0.172
Phenylalanine	0.50	4.9	0.141	0.41	4.4	0.188
Threonine	0.67	6.6	0.190	0.56	6.0	0.257
Tryptophane	0.52	5.1	0.147	0.43	4.6	0.198
Valine	0.55	5.4	0.156	0.46	4.9	0.211
Totals	10.11	99.7	2.888	9.37	100.0	4.307
Total N of the unnatural isomers of the 7 racemic forms 1.675						

The essential amino acids in corn germ protein ( $N \times 5.7$ ) according to the analysis made in this laboratory accounted for only 62.2% of the total nitrogen, and the nitrogen of these natural acids in the average amount ingested by the squad daily was only 2.888 gm while the nitrogen of the natural isomers in the average daily ingestion of the amino acid mixture was 4.307 gm. The partial contribution of each acid in the two diets is found in columns 4 and 7 of table 5. The percentage relationship of the nitrogens of the essential amino acids one to another evidently is not so different in the two forms of feeding the natural isomers as is the absolute amount

of the nitrogen in each. Corn germ protein is able to maintain the biological value of 75.5 with considerably less nitrogen in the natural isomer of each essential amino acid than is the mixture of synthetics (assuming that the nitrogen of each unnatural isomer is totally wasted). The comparison is even more favorable to the natural protein on the basis of total absorbed nitrogen of the natural isomers; for the true digestibility of the protein is only 77%, while that of the amino acid mixture is 97% (table 3).

Putting the best face possible on the comparison of a synthetic protein with the natural one which it imitates in all its best qualities, the nutritional economy of the former makes a poor showing.

### *Beefsteak*

This protein offers the advantage of much less alimentary waste. An attempt was made to effectuate the correction of the nitrogen of the unnatural isomers of a mixture imitating its protein, in the same way as was done successfully with the mixture imitating corn germ protein. The correction, however, was calculated and applied in one single period of 5 days — 3 days before adjustment and 2 afterward. Applied to the preliminary period the calculated increase of nitrogen from unnatural isomers necessary to reproduce the B.V. of the meat, on the assumption that all of it would be excreted, was 1.127 gm. The *dl* synthetics were increased accordingly, without changing the basic forms, and the resulting distribution of the natural isomers shown in table 6 was obtained. As may be seen the actual sum of the contributions from all the unnatural isomers fell slightly below the predicted value.

While the (relative) percentage change in the essentials is only 10 to 12% for the basics and 15 to 17% for the *dl* forms (cols. 3 and 5), the partial contribution which each makes to the absorbed nitrogen of all the natural isomers (cols. 4 and 7) is again considerably higher from the artificial than for the natural protein (about 70% for the basics and 30% for *dl* forms). Notwithstanding this, correction having

been made for all the unnatural nitrogen in the amino acid mixture, the former falls several points short of duplicating the biological value of the latter (table 7). If it had been possible to continue the experiment a few days longer, a second and more exact correction could have been made. The inevitable conclusion is that synthetic amino acids are nutritionally uneconomical.

TABLE 6

*Distribution of nitrogen of natural isomers of essential amino acids in beefsteak and in the mixture of synthetic essentials (after adjustment).*

	IN BEEFSTEAK PROTEIN			IN MIXTURE OF SYNTHETICS		
	Gm N/ 100 gm protein	Per cent of total	Gm each in av. absorbed	Gm N/ 100 gm mixture	Per cent of total	Gm each in av. absorbed
Arginine	3.25	32.7	0.709	3.82	36.2	1.206
Histidine	1.19	11.9	0.258	1.40	13.3	0.453
Isoleucine	0.59	5.9	0.129	0.52	4.9	0.164
Leucine	1.39	14.0	0.304	1.25	11.8	0.392
Lysine	1.54	15.5	0.336	1.80	17.1	0.570
Methionine	0.39	3.9	0.085	0.34	3.3	0.110
Phenylalanine	0.30	3.0	0.066	0.27	2.5	0.083
Threonine	0.60	6.0	0.131	0.54	5.1	0.165
Tryptophane	0.20	2.0	0.044	0.18	1.7	0.056
Valine	0.47	4.7	0.104	0.41	4.0	0.132
Totals	9.92	99.9	2.164	10.53	99.9	3.331
Total N of unnatural isomers 1.102						

The failure of the amino acid mixture in this experiment to duplicate the biological value of the protein, even under the most favorable conditions, might be explained by faulty analysis of the hydrolysate or by the fact that the nonessentials, being already formed in the protein and, possibly already in polypeptid combination with essentials to a greater extent than in corn germ, confer an advantage in time saved for their production as well as in conservation of essentials, or finally by the difficulty of computing the correction factor accurately in the middle of a period before all the analytical data were available. There is some reason to believe that the lysine de-

termination adopted was too low, and lysine, as shown in paper II (Murlin, Edwards, Hawley and Clark, '46b) is retained better than any other of the synthetic essentials when fed singly as an adjuvant to egg or soy bean protein. Indeed, as will be shown in a later publication (Hawley, Edwards, Clark and Murlin paper V, '46), this amino acid seems to induce considerable retention of other nitrogen than its own.

That the correction factor, equal to the total nitrogen of the unnatural isomers present in the mixture, was excreted as expected will be seen from the fractionation of the urines. The total nitrogen was partitioned by direct determination

TABLE 7

*Biological value of beefsteak protein and of the amino acid mixture (after adjustment by increase of dl forms according to calculation below). Averages for 8 men.*

	a	b	c	d	e	f	g	h	i	j	k	N-bal.
	Test protein fecal N	No-protein fecal N	(a-b) Fecal waste N	Test protein N eaten	(d-c) Absorbed N	True digestibility %	Test protein urine N	No-protein urine N	(g-h) Urinary waste N	$i \times \frac{100}{e}$	B.V.	gm
Protein	1.057	1.028	0.029	3.889	3.860	99	2.378	2.040	0.338	8.7	91.3	+ 0.454
Amino acid												
Prelim.	1.039	1.028	0.011	3.946	3.935	99	3.411	2.040	1.371	34.8	65.2	- 0.502
Amino acids												
adjusted	1.039	1.028	0.011	4.667	4.656	99	3.689	2.040	1.526	32.8	67.2	- 0.061
Correction for all N of unnatural isomers:					1.102		1.102					
					3.554		2.587	2.040	0.547	15.4	84.6	- 0.061

Calculation for x in formula (1) p. 723:

$$91.3 = \frac{(3.935-x) - ((3.411-x)-2.040) \times 100}{(3.935-x)}$$

$$91.3(3.935-x) = (3.935-x) - ((3.411-x)-2.040) \times 100$$

$$- 91.3x = (-3.411 + 2.040 + .342)100$$

$$91.3x = 102.9$$

$$x = 1.127$$



TABLE 8

*Distribution of the nitrogen of the urine from beefsteak protein and the amino acid mixture imitating it before and after adjustment.*

*All quantities are averages for the squad of 8 men.*

	TOTAL N	UREA N		AMMONIA N		AMINO ACID N		CREATININE N		URIC ACID N		MINED N UNDETER	
		gm	%	gm	%	gm	%	gm	%	gm	%	gm	%
Beefsteak (Aug. 15)	2.545	1.152	45.3	0.355	13.9	0.232	9.1	0.527	20.7	0.148	5.8	0.132	5.2
Am. acid mixture (Aug. 17)	3.404	1.549	45.5	0.619	18.2	0.410 <sup>1</sup>	12.1	0.510	15.0	0.096	2.8	0.219	6.4
Am. acid mixture after ad- justment (Aug. 19 and 20)	3.689	1.476	40.0	0.832	22.6	0.550	15.0	0.522	14.2	0.110	3.0	0.181	5.0

<sup>1</sup> Approximately 43% of this nitrogen or 27% of the nitrogen of unnatural isomers fed was found by Dr. Bartlett to be of the *d* form.

of urea, ammonia, amino acids, creatinine and uric acid, and, by difference, of the undetermined portion, on one or two of the last days of the beefsteak period, the preliminary amino acid period and the final or adjusted amino acid period (table 8).<sup>8</sup> Noticeable differences between the beefsteak and amino acid periods are found in all columns except that for the absolute amounts of creatinine. The increases in urea and, still more, in the urea plus ammonia fraction, both absolute and relative, are significant. The sum of these two in the two amino acid periods is 63.7 and 62.6%, respectively, of the total nitrogen, while in the beefsteak period it is only 59.2%. Increasing ammonia reflects the greater acidity of the final mixture over the preliminary one and of the latter over that of the natural digestive products. The weight of amino acid nitrogen increases more rapidly from period to period than does the total nitrogen, as shown by the advancing percentages.

<sup>8</sup> Results were available for only a single day of the first two periods. For this reason a small discrepancy will be found between the total nitrogens of the urines of these periods in this table and in table 7.

This of course must mean that more undeaminated amino acids are passing through the kidney as these already digested products reach the blood stream in greater concentration.

Diminishing percentages of creatinine and uric acid nitrogen are expected because, whereas the beefsteak contains definite precursors of both products, creatine and nucleic acids, the amino acid mixture contained only arginine as a possible precursor of creatine. It was not sufficient apparently to overbalance the preformed creatine of the beefsteak.

But it is the sum of the first three fractions with which we are principally concerned, i.e., the sum of the deaminated and undeaminated amino nitrogens appearing in the urine. How well does the difference between these sums for the beef protein period and the final amino acid period agree with the correction factor representing the nitrogen of all the unnatural isomers of the *dl* forms in the final period? The sum for the former is 1.739 gm per man; for the latter it is 2.858 gm per man; and the difference is 1.119 gm. The correction in table 7 is 1.102. The nitrogen intake was increased in the final amino acid period over the beefsteak period by only 0.778 gm per man per day (table 7), but the nitrogen of the *dl* forms amounted to 2.204 gm, and that of the unnatural isomers in this 1.102 gm. It is unbelievable that the agreement between the sum of the nitrogens of the unnatural isomers in the total absorbed nitrogen and the sum of the extra nitrogens in the three fractions of the urine can be accidental. To the writers it means that the unnatural isomers are not available for retention when sufficient amounts of the natural ones are present.

#### SUMMARY AND CONCLUSIONS

In ten comparisons in human subjects of the proteins of whole egg, yeast, cottonseed meal, corn germ flour, beefsteak and haddock, with mixtures of the essential amino acids, compounded in the proportions one to another in which they occur in the proteins and supplying as much nitrogen as the pro-

tein, none of the mixtures possessed a biological value so high as that of the protein by from 10 to 40%.

The mixtures contained arginine, histidine and lysine in the natural *l* (+) form and as the monohydrochloride salt. All the other acids isoleucine, leucine, methionine, phenylalanine, threonine, tryptophane and valine were available in sufficient amounts only as the *dl* synthetic products.

In two experiments based on the hypothesis that if sufficient amounts of the natural isomers of the *dl* forms were supplied the unnatural isomers would be wasted, it was possible to duplicate closely the biological value of the proteins. A preliminary experiment was required to furnish data for calculation of the amount of nitrogen from unnatural isomers which would need to be excreted and subtracted from both sides of the balance sheet to produce the B.V. of the protein. Supplying these amounts in the *dl* isomers in a second experiment and deducting all the nitrogen of the unnatural isomers from both absorbed and urinary nitrogen of the amino acid period gave the predicted B.V. in one experiment and approached it in the other.<sup>9</sup> Correction in this manner for nitrogen representing no nutritional synthetic value has been practised regarding the purine compounds in beverages and would be in order in the case of medicinal agents or urea administered in the course of an experiment as a test substance for kidney function. The unnatural isomers in these experiments insofar as they escape deamination, belong to this class of dispensable compounds; while insofar as they are deaminated and can be recognized as contributing extra nitrogen to the urea and ammonia fractions of the urine, they are in the same class as nonessential amino acids.

The conclusion follows, that the use of *dl* synthetic amino acids, even those of the essential group, is nutritionally uneconomical.

<sup>9</sup> A third experiment (periods 6-iii and iv of table 1) gave the predicted B.V. precisely, but because it was based on earlier and less satisfactory analyses of the proteins concerned it is not reported in detail.

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# STUDIES ON THE COMPARATIVE NUTRITIVE VALUE OF FATS

## VII. GROWTH RATE WITH RESTRICTED CALORIES AND ON INJECTION OF THE GROWTH HORMONE <sup>1</sup>

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THREE FIGURES

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The rate of growth has usually been considered as an index of the nutritional value of a food. Thus, the food or foodstuff which promotes the more rapid growth has been considered to have the higher nutritive value. Schantz, Elvehjem and Hart ('40) originally reported that butter fat possesses a specific nutritive property not present in vegetable fats since greater growth is obtained in weanling rats over 6-week periods on diets of homogenized liquid skim milk and butter fat than when coconut, corn, cottonseed or soy bean oil was used in place of butter. The greater rate of growth observed by these investigators was always associated with a greater food consumption.

Deuel, Movitt, Hallman and Mattson ('44) found no differences in the growth rate of weanling rats fed a mixture of mineralized skimmed milk powder, vitamin supplements and

<sup>1</sup> This work was carried out under a research grant from The Best Foods, Inc. The authors wish to acknowledge the helpful advice of Prof. Anton J. Carlson of the University of Chicago, of Prof. Arthur W. Thomas of Columbia University and of Dr. H. W. Vahlteich of The Best Foods, Inc., during the course of the experiments.

fat, irrespective of whether butter or margarine fat, corn, cottonseed, olive, peanut or soy bean oil was the fat employed. In these experiments not only were the increases in body weight similar in the various dietary groups, but also bone growth was shown to be identical. No appreciable differences in food consumption were noted on the various diets. The specific effect of the saturated fatty acid fraction of butter on growth as postulated by Schantz, Elvehjem and Hart ('40) has not been confirmed (Henry, Kon, Hilditch and Meara, '45; Jack, Henderson, Reid and Lepkovsky, '45). Also the increased requirement of premature rats for butter fatty acids has been denied by Zialcita and Mitchell ('44) and by Deuel and Movitt ('45).

It is probable that flavor plays an important role in the amount of food consumed and hence indirectly on the extent of growth. Rats were found to prefer a butter-skimmed milk powder diet to one in which a bland vegetable fat was employed (Deuel and Movitt, '44). Furthermore, with two diets containing the same vegetable fat, the rat prefers one to which diacetyl or commercial butter flavor has been added to the extent of 1.2 parts per million to one which is unflavored. Although differences in food consumption and in growth were not noted in the earlier tests (Deuel et al., '44) for the groups receiving the butter diet or those containing the vegetable fats, it is probable that this flavor preference may account for the higher food consumption with the resultant superior growth that has in some cases been reported on the butter diets where ad libitum feeding was employed.

On artificial diets containing 48% of lactose, Boutwell, Geyer, Elvehjem and Hart ('44) reported a lower growth associated with a markedly reduced food intake when corn oil was the fat used as compared with tests where butter was employed. When diacetyl was added to the corn oil, food consumption was slightly increased and the difference between the butter and corn oil group decreased by 40%. Although admitting that off flavor or rancidity will have an adverse effect on food consumption, these authors state: .

On purified diets the true explanation for the superior growth-promoting value of one substance over another must lie in the existence of specific compounds in that superior nutrient which results in a more favorable physiological response such that the animal grows at a faster rate and hence consumes more ration.

In the present tests the extent of growth has been augmented by the injection of growth hormone. If any essential component were lacking in sufficient amount in the vegetable fat diets to permit such augmented growth, such a deficiency should then become apparent. On the other hand, the response of rats to diets given in amounts insufficient for normal growth has also been followed in order to determine whether any differences in efficiency of the fat obtains under such conditions.

#### EXPERIMENTAL

Twenty-one-day weanling albino female rats of the Sprague Dawley strain were used. There were 10 rats in each group. In the experiments with restricted food intake, the rats were given a daily quota of 60% of the daily food consumption of control rats receiving the butter diet where ad libitum feeding was employed. The amount of food fed daily during each week, respectively, of the 9-week period of restricted food intake was as follows: 3.1, 4.4, 5.3, 5.6, 5.7, 6.2, 6.0, 6.2, and 6.2 gm. Food was given ad libitum from the tenth to twelfth weeks. The diets were approximately isocaloric and contained optimum quantities of the B vitamins, liver concentrate, and fat-soluble vitamins in addition to those in the skimmed milk powder. The composition of the diets is recorded in table 1.

The diets employed in the hormone tests were similar in composition without the addition of the added vitamins to the skimmed milk powder.

A growth hormone powder prepared from the anterior pituitary gland which assayed 1700 rat (growth) units per gram was used in these experiments.<sup>2</sup> A solution was prepared

<sup>2</sup> This was kindly furnished to us by Dr. Oliver Kamm, Parke, Davis and Co., Detroit, Michigan. Dr. Kamm stated that the figures for potency were based on the usual biological method for determination.



from the powder at frequent intervals by addition of an alkaline aqueous solution, adjustment of the pH to 8.5 and filtration of the undissolved material. Normal butyl alcohol

TABLE 1

*The composition of diets used in studies where food intake was restricted.*

COMPONENT		DIET 55b	DIET 55c
Mineralized skimmed milk powder <sup>1</sup>		70.3	66.2
Added minerals (per 100 lbs.)			
MnSO <sub>4</sub> ·7 H <sub>2</sub> O	3.455 gm		
CuSO <sub>4</sub> ·5 H <sub>2</sub> O	3.340 gm		
Fe·C <sub>12</sub> H <sub>10</sub> O <sub>7</sub> ·3 H <sub>2</sub> O	45.557 gm		
Added vitamins (per 100 lbs.) <sup>2</sup>			
Thiamine hydrochloride	1.27 gm		
Riboflavin	2.54 gm		
Pyridoxine	1.27 gm		
Calcium pantothenate	19.55 gm		
Choline chloride	77.40 gm		
Liver concentrate <sup>3</sup>	2390.00 gm		
Butter or margarine		29.7	
Added supplements per 1000 gm fat <sup>4</sup>			
Carotene	8.6 mg		
Vitamin A concentrate <sup>5</sup>	142.0 mg		
Vioosterol	862.0 mg		
α-tocopherol	10.7 mg		
Commercial butter flavor	4.0 mg		
Vegetable oils (corn, cottonseed, peanut, soy bean, commercial hydrogenated fat)			25.6
Supplements as above			
Water			8.2
Caloric value (cal./100 gm)		480	476

<sup>1</sup> Challenge spray dried powder.

<sup>2</sup> This would give approximately the following in 10 gm of food; thiamine 200 μg; riboflavin, 400 μg; pyridoxine, 200 μg; calcium pantothenate 3 mg; and choline chloride 12 mg.

<sup>3</sup> Wilson 1 : 20 liver concentrate. This gives 0.37 gm per 10 gm food.

<sup>4</sup> Calculated to give the following amounts per 10 gm of food; carotene 25.5 μg, vitamin A, 84.5 I.U.; viosterol, 25.6 U.S.P. XI units; α-tocopherol, 31.8 μg.

<sup>5</sup> Vitamin A concentrate containing 200,000 I.U. per gm.

was used as a preservative in 1% concentration. Placebo solutions prepared in a similar manner without the growth hormone were used with the control groups. The solutions were injected intraperitoneally in 0.1 ml doses daily (6 days weekly).

Two series of tests were carried out at approximately 6-month intervals. In the first series, which consisted of five rats in each group (butter, cottonseed, margarine and soy bean diets without and with growth hormones), all animals survived and were in excellent condition throughout the test. In the second series a few of the rats in each group lost weight over several weeks after which some partially recovered. This occurred chiefly with the rats injected with hormone although in several instances it was found with the control rats. All rats of the litters so affected have been dropped from consideration. The results on corn oil were carried out only on the second series.

#### RESULTS

The average increases in weight of weanling rats receiving the various diets in restricted amounts during a 9-week period are presented in figures 1 and 2. Although weekly weighings were made, the averages are given only for the first, third, sixth and ninth week for the restricted period and for the tenth, eleventh and twelfth week during which ad libitum feeding was carried out. The data are for ten rats in each group.

The average body weight of the groups of rats at the start of the tests varied from 40.7 to 42.0 gm. At the end of 9 weeks of the restricted diets, the average total gain of the rats was as follows: butter (1)  $92.5 \pm 3.5$  gm; corn oil (2)  $95.5 \pm 3.3$ ; cottonseed oil (3)  $103.3 \pm 2.5$ ; margarine (4)  $90.7 \pm 0.7$ ; peanut oil (5)  $97.8 \pm 3.3$ ; soy bean oil (6),  $102.4 \pm 2.1$  and the commercial hydrogenated fat (7),  $89.2 \pm 2.4$ .

There was an immediate response to the ad libitum feeding with a marked increase in the growth rate. After the 3 weeks of ad libitum feeding, the total increase in weight had reached

the following figures: (1) 129.7, (2) 122.1, (3) 137.7, (4) 124.3, (5) 132.9, (6) 138.8 and (7) 124.5 gm.

The average gains in weight of rats receiving the injections of growth hormone daily and the control animals are shown in figure 3.

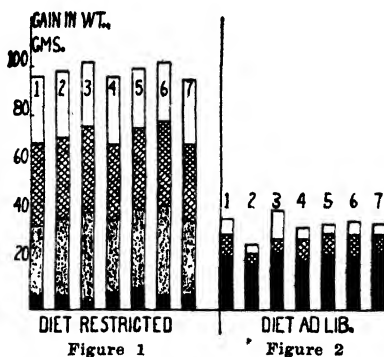


Fig. 1 The average gain in weight of female rats after 1 week (solid black), after 3 weeks (stippled), after 6 weeks (cross-hatched) and after 9 weeks (to top of blank space) on the diets containing the following fats and restricted in calories: 1, butter; 2, corn oil; 3, cottonseed oil; 4, margarine; 5, peanut oil; 6, soybean oil and 7, a commercial hydrogenated fat.

Fig. 2 The average total gain in weight of female rats recorded in figure 1 for tenth to twelfth weeks only where ad libitum feeding was employed. The gain for the first week is in solid black, after 2 weeks (cross-hatched) and after 3 weeks (to top of blank space).

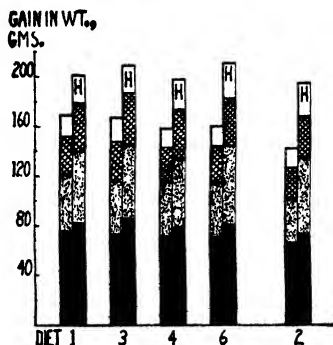


Fig. 3 The average gain in weight of female rats after 3 weeks (solid black), after 6 weeks (stippled), after 9 weeks (cross-hatched) and after 12 weeks (to top of blank space) on the following diets: 1, butter; 3, cottonseed oil; 4, margarine; 6, soy bean oil and 2, corn oil. The experiments on diet 2 were not all carried out simultaneously with the others. The columns marked "H" at the top are for the rats injected with growth hormone throughout and the other column was for the placebo-injected controls.

The average food intake and the relative efficiency of the food in the building of body tissue are summarized in table 2.

The relative efficiency in the utilization of the ingested food (gain in weight  $\times 100$ : cal. consumed) is markedly higher in all cases where the growth hormone was administered. The increased efficiency (in per cent) in the groups injected with growth hormone as contrasted with the placebo-injected controls, was as follows: butter diet, 18.5; cottonseed oil diet, 23.8; margarine diet, 22.3; soy bean oil diet, 14.6; corn oil diet, 29.0.

TABLE 2

*Summary table showing average gain in weight, average food consumption and ratio of increase in weight to calories consumed.*

FAT IN DIET	NUMBER OF RATS	TOTAL GAIN IN WEIGHT	TOTAL FOOD CONSUMED	TOTAL CALORIES CONSUMED	EFFICIENCY <sup>1</sup>
<div style="display: flex; justify-content: space-around; align-items: center;"> <div style="text-align: center;"> <i>gm</i> Rats receiving         </div> <div style="text-align: center;"> <i>gm</i> placebo injection         </div> </div>					
Butter	10	169.7	712.8	3420	4.97
Cottonseed	10	167.0	755.8	3590	4.65
Margarine	7	158.1	717.4	3410	4.63
Soybean	9	159.9	683.8	3250	4.92
Corn	9	142.1	668.2	3175	4.47
<div style="display: flex; justify-content: space-around; align-items: center;"> <div style="text-align: center;">Rats receiving 10 units of</div> <div style="text-align: center;">growth hormone daily</div> </div>					
Butter	9	201.3	711.3	3415	5.89
Cottonseed	9	208.6	761.0	3625	5.76
Margarine	8	197.4	734.7	3490	5.66
Soybean	8	211.0	786.9	3740	5.64
Corn	9	194.4	710.3	3375	5.77

<sup>1</sup>  $\frac{\text{Gm increase in weight}}{\text{Cal. consumed}} \times 100.$

## DISCUSSION

No appreciable variation in the limited rate of growth of rats was found over a 9-week period irrespective of whether the skimmed milk diet contained a butter, a margarine, a commercial hydrogenated fat, or corn, cottonseed, peanut, or soy bean oil. Moreover, during a 3-week period of ad libitum feeding following the restricted period, the response of the rats by increased growth was immediate in all cases.

There is no indication from these tests that inclusion of butter in low calorie diets gives any superior response to that of vitamin-fortified vegetable oils, a margarine or a commercial hydrogenated fat.

In the experiments where growth hormone was injected, it was found that all groups were able to respond by an increased growth. The actual increase in growth in the corn oil tests (52.3 gm) was greatest in percentage although a similar response was obtained with the soy bean group (51.1 gm) while the increase in the butter group was only 31.6 gm. The somewhat lower gain in weight of the corn oil control group is probably to be ascribed to the fact that the experiments were carried out on only the second series of tests while with the other fats the tests were made with both series. The growth of the rats in all groups was lower in the second series of tests.

Although no appreciable variation was noted in the efficiency with which the different diets were utilized in the control tests, there was in all cases an increase in the efficiency of the utilization of the diets in the hormone-injected rats. The energy value of the injected hormone preparation itself was insignificant. If one assumes that 50% of the solids were present in the final solution as well as 1 mg of butyl alcohol in the daily quota, this would account for a maximum of 11 cal.; this quantity is inappreciable in comparison with the total caloric intake over the 84-day period which approximated 3500 cal. This increased efficiency in the hormone-treated rat is in line with the earlier results of Lee and Schaffer ('34) and of Marx et al. ('41-'42) who reported greater growth with an identical calorie intake.

It is our opinion that an excellent method to test the nutritive value of a diet is by its ability to support the additional growth occasioned by the injection of growth hormone. If the diet is inadequate, not only would one expect little additional growth on the injection of the hormone but also one might expect dietary failure sooner. Such a result has been reported by Ershoff and Deuel ('45) where a shorter length

of life obtained in rats deficient in vitamin A when hormone was injected than in the untreated controls. On the other hand, when vitamin A was also administered along with the basal diet, the growth was greater than in groups of rats on a similar intake of vitamin A without the growth hormone. The fact that a marked response to the growth hormone resulted in all groups in the present experiments would indicate that the diets were nutritionally satisfactory. This augmented dietary requirement can be as readily satisfied by vegetable fats as by butter.

#### SUMMARY

When rats were fed diets of mineralized skimmed milk powder and vitamin-fortified fats at a level of 60% of the ad libitum intake over a period of 9 weeks following weaning, the rate of growth was identical irrespective of whether the fat employed was a butter, a margarine, a commercial hydrogenated fat, or corn, cottonseed, peanut or soy bean oil. Moreover, there is no indication that differences exist in the ability of the rats to respond with increased growth during a 3-week period of ad libitum feeding following the period of restricted food intake in any dietary groups.

When growth hormone was injected, the augmented growth was as great or greater with the rats receiving the vegetable fat diets as with those receiving the butter diet. Since not only does increased growth not occur when growth hormone is injected in rats receiving deficient diets (i.e., vitamin A-free) but also the period of survival is decreased, the present results are interpreted as indicating that the various vegetable fats and margarine have an ability equal to butter in supporting such added growth requirements.

When growth hormone was injected, a greater efficiency in the utilization of the foodstuffs for growth was found.

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# STUDIES ON THE COMPARATIVE NUTRITIVE VALUE OF FATS

## VIII. THE FAILURE OF ETHER EXTRACTION TO LOWER THE NUTRITIVE VALUE OF SKIMMED MILK POWDER IN DIETS CONTAINING VARIOUS VEGETABLE FATS <sup>1</sup>

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### ONE FIGURE

(Received for publication January 14, 1946)

In the first report of the present series of papers (Deuel, Movitt, Hallman and Mattson, '44) it was shown that no differences occurred in the rate of growth of male or female rats fed on diets of unextracted mineralized skimmed milk powder and vitamin-fortified fats irrespective of whether a butter, a margarine or corn, cottonseed, olive, peanut or soybean oil was the fat employed. In the original paper of Schantz et al. ('40), where the most decisive differences between the butter and vegetable fat groups were reported, unextracted liquid skimmed milk was used. In their later tests (Boutwell et al., '43) skimmed milk powder was used which had been extracted with diethyl ether for four 8-hour periods during which it was subjected to constant agitation.

In an editorial discussion on the nutritive value of butter fat (Anonymous, '44), a possible explanation was offered

<sup>1</sup> This work was carried out under a research grant from The Best Foods, Inc. The authors wish to acknowledge the helpful advice of Prof. Anton J. Carlson of the University of Chicago, of Prof. Arthur W. Thomas of Columbia University and of Dr. H. W. Vahlteich of The Best Foods, Inc., during the course of the experiments.



for the divergent results of the Wisconsin group and those of Deuel et al. ('44), namely, that the vegetable oil diets in the latter tests actually contained some butter fat since the skimmed milk powder used had 1.1% of residual fat. Boutwell et al. ('45) have also recently made a similar implication when they state that "... Deuel et al. (3, 4, 5) fed weanling rats an unextracted skimmilk powder ration . . ."

The amount of residual fat available to the rat from the unextracted skimmed milk powder seems far too small to be of consequence. Based on an average daily food intake of 10 gm, the total butter fat from the skimmed milk powder containing 1.1% lipid would amount only to 77 mg. This would give a maximum of 28 mg of saturated fatty acids of the fraction ( $C_{16}$ - $C_{20}$  (Jack et al., '45)), which consists of palmitic and stearic acid. This is the fraction of butter which is considered by Schantz, Elvehjem and Hart ('40) to contain the fatty acids having the specific nutritive value. It is difficult to see how in addition to palmitic and stearic acids, there could be any appreciable quantity of hitherto unidentified fatty acids in sufficient amount to result in any improvement in the nutritive value of the diet. The optimum quantity of methyl linolate required for growth is probably about 100 mg daily.<sup>2</sup> It should also be mentioned that Jack et al. ('45) and Henry et al. ('45) have failed to demonstrate any specific nutritive value in this saturated fatty acid fraction of high molecular weight or in any other fraction of butter fat.

The present tests were designed to determine how completely fat could be removed from skimmed milk powder by the treatment employed by Boutwell et al. ('43) and also to ascertain whether under the experimental conditions which we have employed any differences would be observed in the growth of weanling rats fed such extracted skimmed milk powder combined with a butter, or a margarine or the vegetable fats instead of the unextracted product combined with these fats.

<sup>2</sup> Personal communication from Dr. G. O. Burr.

## METHODS AND RESULTS

*Experiments on extraction of fat from skimmed milk powder*

In order to determine the effectiveness of diethyl ether in removal of the fat, approximately 700 gm of skimmed milk powder<sup>3</sup> was suspended in 3,000 ml of redistilled U.S.P. diethyl ether and vigorously agitated for 8 hours with a large paddle operated by an air stirrer so that the solid was kept in suspension. The ether was filtered off, the skimmed milk powder suspended in fresh ether, and the procedure repeated eight times. Samples of the skimmed milk powder were analyzed after each extraction by the Roesse-Gottlieb procedure. In one series the fat removed in the ether was also determined in each case. Extraction was also carried out with diethyl ether in a special Soxhlet apparatus<sup>4</sup> for three successive 24-hour periods followed by two 24-hour extractions with 95% ethyl alcohol. A combined diethyl ether-ethyl alcohol extraction over 48 hours was also used. The results are summarized in table 1.

In the tests recorded in table 1, only 12, 26 and 31% of the fat were removed by four successive 8-hour extractions with diethyl ether. Even after eight extractions of the skimmed milk powder, only 21, 38, and 48% were removed in different tests. This value almost exactly coincides with that found by 72 hours of continuous extraction of the skimmed milk powder on the Soxhlet apparatus using diethyl ether. A further extraction with warm ethyl alcohol also in the Soxhlet apparatus reduced the residual fat to as low as 0.35%. The most effective and quickest removal of fat would seem to be by the combined simultaneous extraction on the Soxhlet with alcohol and ether. After 48 hours extraction on the Soxhlet apparatus, the fat was reduced to 0.29%.

The ineffectiveness of diethyl ether as an extraction agent is further indicated by the results obtained on different

<sup>3</sup>Challenge spray dried skimmed milk powder was used.

<sup>4</sup>A specially built Soxhlet having an extraction chamber of 4-liter capacity built by Mr. E. Greiner was used.

batches of extracted skimmed milk powder used in the feeding tests. The residual fat content was found to be 0.84, 0.77, 0.70 and 0.61% in four different lots compared with a value of 0.87% in the unextracted sample.

TABLE 1

*The effectiveness of continuous stirring with diethyl ether and of Soxhlet extraction with ether, alcohol or an alcohol-ether mixture in removing lipid from skimmed milk powder.*

AIR STIRRER (DIETHYL ETHER)					SOXHLET				
Time of extraction	Fat content				Time of extraction	Solvent	Residual fat content		
	Sample 1 Residual	Sample 2 Residual	Sample 3				Sample 1	Sample 2	Sample 3
			Residual	Ex-tracted					
hours	%	%	%	%	hours		%	%	%
0	1.01	1.01	1.00						
8	0.94	0.91	0.93	0.046	24	Ether	0.90	0.96	
16	0.86	0.93		0.039	48	Ether	0.91	0.81	
24	0.83	0.78	0.92	0.032	72	Ether	0.84	0.78	
32	0.75	0.70	0.88	0.027					
40	0.74	0.71	0.84	0.018	24	Alcohol <sup>1</sup>	0.45	0.54	
48	0.73	0.66	0.82	0.020	48	Alcohol	0.35	0.56	
56	0.67	0.60	0.80	0.015	24	Alcohol-ether <sup>2</sup>			0.34
64	0.63	0.53	0.79	0.015	48	Alcohol-ether			0.29
Total				0.212					

<sup>1</sup> Ether extraction for 72 hours followed by alcohol extraction.

<sup>2</sup> Alcohol-ether mixture used of such proportions so that ether would fill approximately half of the extracting chamber. The alcohol would then be distilled over to fill the chamber to the point of siphoning giving a 1:1 mixture of the solvents at that time.

*Feeding experiments with diets made up of extracted skimmed milk powder and different fats*

Feeding experiments were made with weanling male and female rats on diets of extracted skimmed milk powder to which was added a butter, a margarine, or corn, cottonseed, peanut or soybean oil. The diets and other experimental

procedures were the same as those in our original experiments (Deuel et al., '40) except that rats of the Sprague Dawley strain were used. Ten rats were used in each group. The gains in weight at 3, 6, 9, and 12 weeks are given in figure 1 and a summary of the data on efficiencies of the various diets is reported in table 2.

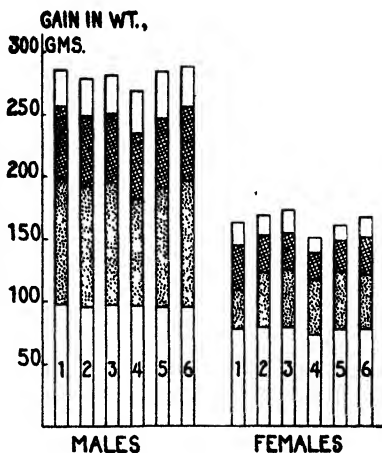


Fig. 1 The average gain in weight of male and female rats for 3 weeks (lower blank space), 6 weeks (stippled), 9 weeks (cross-hatched) and 12 weeks (upper blank space) is given for the following diets: 1, butter; 2, corn oil; 3, cottonseed oil; 4, margarine; 5, peanut oil; 6, soybean oil.

No differences in the rate of growth or in the total gain were noted in the diets with extracted skimmed milk powder irrespective of whether the fat used was a butter, a margarine or corn, cottonseed, peanut or soybean oil. The efficiency of utilization of the foodstuffs is the same within experimental error in all cases.

#### DISCUSSION

The divergent results obtained by Deuel et al. ('44) and Boutwell et al. ('43) cannot be ascribed to the use of unextracted skim milk powder by the former investigators. The extraction procedure on the skimmed milk powder employed by the latter group (diethyl ether for four successive

8-hour periods) removes only a small fraction of the total residual lipids in the milk powder. In addition, using a similar strain of rats employed by the Wisconsin group, it was shown

TABLE 2

*The food consumption and efficiency of utilization of diets of mineralized-extracted skimmed milk powder with different fats when fed to rats over a 12-week period.*

DIET	BODY WEIGHT		TOTAL GAIN <sup>1</sup>	TOTAL FOOD	TOTAL CALORIES	EFFICIENCY <sup>2</sup>
	Start	End				
	gm	gm	gm	gm		
Male rats						
Butter	42.5	328.1	285.6 ± 5.5	968.9	4545	6.28
Corn oil	42.6	322.2 (9)	278.9 ± 5.4	987.6	4635	6.02
Cottonseed oil	42.1	323.6	281.5 ± 6.6	972.2	4560	6.10
Margarine	42.4	311.1	268.7 ± 3.4	929.1	4357	6.17
Peanut oil	42.1	325.7 (9)	284.3 ± 5.9	979.2	4600	6.19
Soybean oil	42.1	331.7	289.4 ± 5.9	999.4	4688	6.18
Female rats						
Butter	42.8	206.2	163.4 ± 6.7	732.7	3436	4.75
Corn oil	43.3	211.8	168.5 ± 4.8	768.1	3602	4.68
Cottonseed oil	42.6	215.3	172.7 ± 5.8	795.2	3729	4.62
Margarine	43.9	195.0	151.1 ± 7.5	744.0	3489	4.33
Peanut oil	43.3	204.2	160.9 ± 3.9	788.5	3698	4.34
Soybean oil	42.9	210.3	167.4 ± 4.6	807.0	3788	4.42

<sup>1</sup> Including the standard error of the mean calculated as follows:

$$\sqrt{\frac{\sum d^2}{n}} / \sqrt{n}$$

where "d" is the deviation from the mean and "n" is the number of observations.

<sup>2</sup> Ratio of  $\frac{\text{gm gain} \times 100}{\text{cal. consumed}}$

that no differences in the rate of growth occur between butter and the vegetable fats when extracted skimmilk powder is used.

#### SUMMARY

Residual fat is removed from skimmed milk powder only to the extent of 12 to 31% by four successive 8-hour extractions with diethyl ether and to 21 to 48% when extraction is continued for four additional 8-hour periods when the mixture is constantly agitated. Continuous extraction for 72 hours with diethyl ether on a Soxhlet apparatus removes about 20% of the residual lipids while extraction with ethyl alcohol resulted in a lowering of 35% of the original content. The most effective extraction was made by simultaneous extraction with alcohol and ether.

No differences in rate of growth or in the total ultimate gain in weight over a 12-week period was observed when weanling male or female rats were fed extracted skimmed milk powder mixed with fat, irrespective of whether a butter or a margarine or corn, cottonseed, peanut or soybean oil was the fat used.

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# THIAMINE, RIBOFLAVIN, NICOTINIC ACID, PANTOTHENIC ACID AND ASCORBIC ACID CONTENT OF RESTAURANT FOODS <sup>1</sup>

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The vitamin content of the human dietary has been the subject of considerable study and speculation within recent years. Indirect information is obtainable from the analysis of vitamins in uncooked foods (Waisman and Elvehjem, '41; Cheldelin and Williams, '42). More direct studies have reported cooking losses and vitamin contents in foods prepared under controlled conditions which might be duplicated in household practice (Lane, Johnson and Williams, '42; Cheldelin and Williams, '43; McIntire, Schweigert and Elvehjem, '43; Oser, Melnick and Oser, '43).

Similar investigations of foods eaten in restaurants and in various institutions have emphasized the losses incurred during preparation and service of foods (Eakin and Gerrard, '43; Heller, McCay and Lyon, '43; Nagel and Harris, '43; Peterson, '44; Wertz and Weir, '44; Koch et al., '45). Less information has been published, however, regarding the amounts of B vitamins which are actually available to the

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restaurant-going public. The present work was undertaken for this purpose, as well as to compare the vitamin contents of foods from different grades of restaurants. It includes analyses for the thiamine, riboflavin, nicotinic acid, pantothenic acid and ascorbic acid contents of foods obtained from three Corvallis establishments representing three price groups.

This study is not extensive. Only one sample of each food was analyzed, and no figures on the variation among samples are available. The data, however, give no evidence of important differences in the vitamin contents of foods from the three restaurants, and show that adequate vitamin intakes may be obtained by judicious selection of meals.

#### EXPERIMENTAL

The foods for analysis were obtained from each restaurant at mealtime, so as to secure samples as served. They included foods comprising the regular meals as well as several popular a la carte items such as sandwiches, chili, etc. Individual servings of each item were immediately brought to the laboratory, where they were weighed and homogenized with several volumes of water in a Waring blender to obtain representative samples.

Thiamine and pantothenic acid assays were performed on enzymatically digested samples, using *Lactobacillus fermenti* for thiamine (Sarett and Cheldelin, '44) and *Lactobacillus arabinosus* for pantothenic acid (Hoag, Sarett and Cheldelin, '45). Samples for riboflavin analyses were autoclaved with 0.1 N HCl for 15 minutes (Strong and Carpenter, '42) and assayed by the method of Snell and Strong ('39), using additional amounts of glucose and acetate in the medium as suggested by Stokes and Martin ('43). For nicotinic acid analyses, aliquots were autoclaved for 30 minutes in 1 N H<sub>2</sub>SO<sub>4</sub>, and assayed by the microbiological method of Sarett, Pedersen and Cheldelin ('45). The foods analyzed for ascorbic acid were blended with seven volumes of 1% metaphosphoric acid and assayed by the 2,6-dichlorophenolindophenol method of Loeffler and Ponting ('42). In the first

groups of foods studied, ascorbic acid analyses were carried out on most of the samples collected. Since most of the cooked foods contained less than 10  $\mu\text{g}$  per gm, subsequent analyses were performed only on fruits, juices and some of the vegetables.

### RESULTS

Values for the thiamine, riboflavin, nicotinic acid, pantothenic acid, and ascorbic acid content of the foods from the three restaurants are presented in tables 1, 2, and 3 in terms of micrograms or milligrams per individual portion. Restaurant A (table 1) is a hotel coffee shop, which represents a "better class" establishment in a small town; B (table 2) is a moderate restaurant which caters to campus personnel, and C (table 3) serves food lower in price and of seemingly poorer quality. The sampling of all three restaurants was performed during 1944 and 1945, when rationing may have had some influence upon the quality of foodstuffs obtainable. No marked differences were found among the vitamin contents of the foods served at the three restaurants.

Since each of these menus represents the food served at one particular meal and affords many different meal combinations, only approximate estimates may be made of an individual's vitamin intake at each restaurant. Also, food consumption at breakfast varies greatly, and individual likes and dislikes for certain food items (particularly bread) may influence the values obtained. At restaurant A the approximate ranges of intake of thiamine, riboflavin, nicotinic acid, and pantothenic acid for the various daily combinations of meals possible are 0.6-1.8, 1.4-2.3, 8-26 and 3.6-8.3 mg, respectively. For restaurant B these figures are 0.9-1.8, 1.2-2.1, 10-25, and 4.2-10.3 mg, respectively. At C the comparable amounts of the vitamins are 0.7-2.5, 0.7-2.7, 8-30, and 3.1-18 mg, respectively.

The thiamine intake obtainable from a varied diet at these restaurants tends to be lower than the value of about 1.5 mg per day, recommended by the Food and Nutrition Board of the National Research Council ('45), unless some of the thia-

*The vitamin contents of foods served in restaurant A.*

FOODS	WEIGHT	THIAMINE	NICOTINIC ACID	RIBO- FLAVIN	PANTO- THENIC ACID	ASCOBIC ACID
	gm	mg	mg	mg	mg	mg
Breakfast						
Tomato juice, canned	175	60	1800	37	350	35
Orange juice, fresh	170	153	660	41	257	102
Figs, canned	108	11	200	15	75	
Applesauce, canned	113	34	40	12	113	
Grapefruit, canned	157	80	310	17	110	30
Prunes	140	14	64	41	195	3
Cream of wheat, with cream	355		438	114	597	
Oatmeal with cream	360	338				
Pep	32	190	1150	35	150	
Post Toasties	31	68	325	16	62	
Cream for cereal	120	24	108	90	385	
Fried egg (1)	47	36	45	165	850	
Scrambled egg (1)	52	40	36	160	1190	
Boiled egg (1)	47	36	35	150	940	
Sausage	70	450	3400	260	980	
Ham	41	310	1900	125	450	
Bacon	20	52	900	60	335	
Waffle (1)	71	100	960	185	385	
Hot cakes (3)	219	240	2300	370	100	
Toast, 2 slices	46	22	460	28	130	
Doughnuts (2)	81	40	630	40	315	
Lunch						
Macaroni and cheese	185	74	610	315	407	
Sirloin tips, with vegetables	230	69	4840	345	505	10
Hamburger	103	41	4950	206	392	
Salmon, canned	112	5	6280	168	426	
Short ribs of beef, boiled, (183 gm with bones)	90	15	2880	144	135	
Potatoes, mashed	84	42	680	45	296	0
Mixed vegetables — turnip, onion, carrot	182	36	1055	76	310	3.6
String beans	75	23	270	56	53	0
Potato salad	111	33	880	58	520	0
Gelatin salad	60	10	90	23	42	0
Chili, bowl	260	143	2860	197	364	7
Bread, 2 slices	52	25	400	28	140	
Milk, individual bottle	240	132	168	500	1000	
Dinner						
Rice tomato soup	175	10	470	26	88	1.5
Peach and cottage cheese salad	83	10	250	100	100	1
Ham, baked with pineapple	81	610	2200	137	420	
Chicken pot pie	156	94	4370	156	470	
Veal loaf, baked	146	146	4100	340	890	
Halibut, grilled	120	110	9100	100	390	
Link sausage	93	510	2320	150	450	
Potato, boiled	101	60	570	22	300	7
Corn, whole kernel	71	16	500	37	210	3
Shrimp Newberg	176	79	780	210	769	
Crab Louie	275	165	1200	220	1800	
Rolls (2)	67	124	1540	167	210	
Peach pie	187	205	2170	93	225	
Apple pie	138	180	1300	77	165	
Cake, white	73	22	170	62	220	

TABLE 2  
*The vitamin contents of foods served in restaurant B.*

FOODS	WEIGHT	THIAMINE	NICOTINIC ACID	RIBO- FLAVIN	PANTO- THENIC ACID	ASCOORBIC ACID
	gm	μg	μg	μg	μg	mg
Breakfast						
Tomato juice, canned	188	150	1220	28	325	47
Orange juice, fresh	193	280	830	33	465	110
Grapefruit juice, canned	181	132	235	16	200	48
Prunes	119	6	715	20	200	0
Cereal, Ralston type and cream	275	146	2100	173	600	
Corn flakes	33	220	600	15	55	
Bran flakes, 40%	33	220	3100	51	290	
Fried egg (1)	48	57	36	70	1420	
Poached egg (1)	54	75		79	1170	
Bacon	15	114	1100	30	225	
Waffle (1)	105	160	1450	140	800	
Hot cakes (3)	215	290	2800	175	1270	
Toast, 2 slices	50	133	410	23	152	
Lunch						
Celery soup	200	42	200	230	920	
Meat pie	207	75	6300	310	750	
Potatoes, mashed, and gravy	101	73	1100	70	350	0
Green peas	85	40	700	48	102	
Spanish corn	147	57	1250	75	300	
Spinach	119	25	580	130	70	
Turnips, creamed	101	27	300	30	170	
Salad, carrot and cabbage	110	10	210	27	310	8
Apple pie	129	85	890	65	130	
Custard pie	138	27	480	193	1300	
Hamburger sandwich	140	103	3980	137	500	
Milk, individual bottle	240	86	168	370	960	
Dinner						
Cream of potato soup	210	105	900	245	710	
Swiss steak	95	65	4750	145	350	
Dinner steak, rare	116	130	6000	185	830	
Cauliflower	57	49	305	74	350	
String beans	70	28	155	30	45	
Potatoes, mashed, and gravy	98	83	1420	45	340	
Cottage cheese salad	96	35	71	182	200	
Rhubarb pie	152	213	1530	110	190	
Cake, chocolate	67	14	215	35	135	
Ham sandwich, cold	126	315	2950	230	655	

mine rich foods are consumed regularly. Thiamine is readily lost in cooking (Lane, Johnson and Williams, '42; Nagel and Harris, '43) and the interval between cooking and service may also contribute to further losses (Nagel and Harris, '43).

The riboflavin level in these diets is influenced considerably by the amount of milk consumed. The inclusion of one glass of milk per day with an average diet at these establishments

TABLE 3  
*The vitamin contents of foods served in restaurant C.*

FOODS	WEIGHT	THIAMINE	NICOTINIC ACID	RIBO- FLAVIN	PANTO- THENIC ACID	ASCORBIC ACID
	gm	μg	μg	μg	μg	mg
Breakfast						
Tomato juice, canned	167	70	1500	43	234	25
Orange juice, canned	195	154	515	53	350	58
Oatmeal and cream	263	258	445	180	640	
Fried egg (1)	42	63	61	128	1565	
Ham	55	300	5100	204	990	
Bacon	31	195	1750	58	255	
French toast	125	183	1330	332	1100	
Doughnuts	78	90	515	94	300	
Lunch and dinner						
Vegetable soup	132	30	570	25	185	11
Meat loaf	244	180	2500	178	810	
Hamburger	126	176	6500	272	960	
Wieners and sauerkraut	151	74	1620	92	440	
Red snapper	129	135	3640	166	465	
Pork steak	141	815	2900	271	830	
Salmon	103	120	6700	128	710	
Sausage	83	480	2020	155	550	
Oysters, fried	152	595	3800	284	1450	
Liver	113	400	12400	455	8000	
Potatoes and gravy	99	68	740	52	297	6
Turnips	89	62	545	23	115	0
Vegetable salad	70	58	142	34	112	5
Bread, 2 slices	49	115	1360	90	202	
Pumpkin pie	106	61	335	188	550	
Berry pie	137	42	490	41	252	
Hot beef sandwich	193	340	5150		870	
Milk, individual bottle	237	111	152	520	1070	

allows sufficient daily riboflavin intake to satisfy the National Research Council's recommendations of 1.6–2.0 mg ('45).

The nicotinic acid content of these diets, in most combinations, also meets the allowance of 12–15 mg per day recommended by the National Research Council ('45). In addition to the nicotinic acid figures shown above, as much as 1 mg per cup of coffee (which was not included in the present analyses) may be added to the intake (Teply, Krehl and Elvehjem, '45) if it is assumed that the materials in coffee which stimulate the growth of *L. arabinosus* possess antipellagric value.

Since the human requirements for pantothenic acid are not known, the values given for this vitamin cannot be interpreted at present.

The recommended daily intake of 75 mg of ascorbic acid (National Research Council, '45) can only be met in all three restaurants by frequent consumption of orange juice. The only respect in which restaurant C appears to be inferior to the others is in its use of canned orange juice, which is lower in ascorbic acid than fresh orange juice. The extreme lability of ascorbic acid makes restaurant salads and cooked vegetables a poor source of this vitamin. This is particularly evident in the variable values of 0 to 7 mg for different portions of potatoes.

#### SUMMARY

Single portions of food served for each meal at three restaurants were analyzed for thiamine, riboflavin, nicotinic acid, pantothenic acid, and ascorbic acid. Although the food served by the three restaurants varied with respect to cost and attractiveness as served, there were no marked differences in the vitamin content.

From estimates of daily food intake, it was calculated that riboflavin and nicotinic acid requirements were readily met by varied diets in all three restaurants. Thiamine intake on these diets were equal to the recommended daily allowances, if care was taken in the selection of foods. Judging from the data obtained ascorbic acid requirements could be maintained only by the inclusion of fresh fruit juices.

The vitamins most readily lost in cooking and handling of restaurant foods were thiamine and ascorbic acid.

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# THE COMPARATIVE VALUE OF CERTAIN DIETARY PROTEINS FOR HEMOPOIESIS IN THE RAT<sup>1</sup>

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There is adequate evidence demonstrating that, although hemoglobin formation has a "high priority call" on available protein in the organism, the feeding to the rat of a diet containing either an inadequate amount of dietary protein (Orten and Orten, '43; Metcoff, Favour and Stare, '45) or a qualitatively incomplete protein (Hogan, Powell and Guerant, '41; Harris, Neuberger and Sanger, '43; Albanese, Holt, Kajdi and Frankston, '43; and Orten, Bourque and Orten, '45) will result in the development of a mild to moderate chronic anemia. Other investigations have shown the need of an adequate quantity of dietary protein for satisfactory hemopoiesis in the dog (Sturgis and Farrar, '35; Hahn and Whipple, '39) and in man (Bethel, '36; Leverton, McMillan and Peters, '44).

The problem of the comparative value of different commonly consumed dietary proteins for hematopoiesis is raised by the foregoing studies. The scanty information on this subject in the literature is open to criticism because of short feeding periods and errors introduced by the utilization of stored or tissue protein. In order to completely control this factor, it

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appears necessary that the protein to be evaluated must be the only protein the test animal has ingested for a very long period of time, preferably since weaning. The present study was undertaken with this object in mind and the rat was selected as the test animal for obvious practical reasons.

The proteins lactalbumin, casein, dried skim milk, dried beef blood, and a mixture of dried skim milk and dried beef blood were used. These were fed at both an 18% and a 2.8% protein level to groups of weanling rats. The low-protein level was used to exaggerate possible slight differences between proteins which might not be evident at a higher level. Body weights were followed simultaneously so that a comparison of the value of the proteins for somatic growth as well as for hemoglobin formation was possible. After the animals had been on the experiment for approximately 200 days the hematopoietic value of the various proteins was determined by another technique, that of recovery from a standardized, hemorrhagic anemia.

#### PROCEDURE

Male and female rats of the Connecticut Agricultural Experiment Station strain, weighing from 40 to 50 gm at weaning, were used. They were housed in individual, wide-mesh bottom cages. The litters were distributed into the various groups, using 10 to 14 rats in each group. The composition of the diets is given in table 1. The protein content of the various preparations was determined by nitrogen analysis of dried, ash-free samples. The following N-conversion factors were used: dried skim milk, 6.38; dried beef blood, 6.25; dried skim milk-beef blood mixture, 6.34. In the case of lactalbumin and casein, the moisture and ash contents of the samples employed were determined and were allowed for in computing the amounts of these two proteins used in the diets. The amount of dextrin in the diet was altered to allow for variations in the amounts of the protein containing materials used, as shown in table 1. The ratio of dried skim milk to dried beef blood solids in the mixture employed was 7 to 3.

Body weights were recorded weekly and hemoglobin determinations were made at the intervals indicated in table 2. An acid-hematin photoelectric method, repeatedly checked against samples of blood, the hemoglobin content of which had been determined by the oxygen-capacity procedure, was used. Erythrocyte counts were also made when the animals were 120 days of age.

TABLE 1  
*Percentage composition of diets.*

DIET	PROTEIN SOURCE	DEXTRIN	SUCROSE	CRISCO	WESSON'S SALT MIXTURE
1. Diets containing 18% protein					
Lactalbumin	22.5	36.5	10	27	4
Casein	22.5	36.5	10	27	4
Blood solids-milk solids mixture	32.9	26.1	10	27	4
Skim milk solids	50.6	8.4	10	27	4
Beef blood solids	20.0	39.0	10	27	4
2. Diets containing 2.8% protein					
Lactalbumin	3.5	55.5	10	27	4
Casein	3.5	55.5	10	27	4
Blood solids-milk solids mixture	5.1	53.9	10	27	4
Skim milk solids	7.9	51.1	10	27	4
Beef blood solids	3.1	55.9	10	27	4

Vitamin supplements: "Ryzamin B" (no 2) — 200 mg daily.  
 Wilson's liver ext., "B" — 200 mg daily.  
 Haliver oil with viosterol — 3 drops twice weekly.

When the animals fed the 18% protein level and the surviving rats fed 2.8% protein as lactalbumin had been on the experiment for approximately 200 days, the value of the various proteins for hemoglobin formation in response to hemorrhage was determined in the following manner. The hemoglobin content of the blood of the rat was first determined. Then 30% of the calculated blood volume (2.0 ml blood per 100 gm body weight) was removed with the aid of gentle massage from the warmed, oiled tail. An equal volume

of 0.9% physiological saline was immediately injected intraperitoneally for fluid replacement. Hemoglobin determinations were then made 24 hours later and at regular intervals thereafter until the concentration reached the initial, pre-hemorrhage level. The days required for the hemoglobin level to return to the original value were thus determined. The food consumption of the animal was carefully measured during the period of recovery from the hemorrhagic anemia.

Using the data obtained above the following values were calculated in the manner indicated:

$$(1) \text{ Mg Hb removed} = \text{ml blood removed} \times \text{mg Hb per ml.}$$

$$(2) \text{ Mg Hb formed per day} = \frac{\text{Mg Hb removed}}{\text{Days to regain initial Hb value}}$$

$$(3) \text{ Mg Hb formed per day per 100 gm body wt.} = \frac{\text{Mg Hb formed per day}}{\text{Body weight in 100 gm}}$$

$$(4) \text{ Mg Hb formed per day per gm dietary protein consumed} = \frac{\text{Mg Hb formed per day}}{\text{Gm protein ingested daily}}$$

The value of each protein for hemoglobin regeneration was determined two or three times on each animal of a given group and the results were averaged. Successive hemorrhages had no consistent effect on the ability of the animal to form new hemoglobin.

## RESULTS

As is shown by the averaged body weight data given in table 2, lactalbumin and casein fed at an 18% protein level gave a somewhat better rate of growth in male rats than did either skim milk solids or the mixture of skim milk solids and beef blood solids. In contrast, beef blood solids alone, although fed at the same protein level, supported only a very slight increase in body weight. The same differences in growth rate were found in the female rats of the various groups (data not included in table 2).

Of the male animals fed the low level of protein (2.8%, table 2), those receiving lactalbumin were definitely better off than those receiving casein or protein from the other sources. As was to be expected, the rate of growth was only slight in

any case, but all of the lactalbumin-fed rats survived a 180-day experimental period whereas nearly all of the animals receiving casein or the other sources of protein succumbed. The same was true of the female rats in the various groups.

The averaged hemoglobin data, given in table 3, indicate that the various proteins, with the possible exception of blood solids, fed at an 18% protein level, are of about equal value in maintaining a normal hemoglobin level in the growing rat.

TABLE 2

*Average body weights (in gm) of groups of male rats fed various proteins.*

PROTEIN	NO RATS	DAYS OF AGE						
		24	40	55	77	90	120	200
1. 18% Protein level								
Lactalbumin	7	42	131	215	339	394	431	552
Casein	8	43	144	234	389	437	506	572
Blood solids-milk solids mixture	8	44	92	145	252	254	301	415
Skim milk solids	5	43	80	117	188	224	305	438
Beef blood solids	6	44	36	39	47	49	61	62
2. 2.8% Protein level								
Lactalbumin	5	44	47	49	55	59	74	80 <sup>1</sup>
Casein	6	43	42	40	38	37		
Blood solids-milk solids mixture	6	46	42	41	39	37		
Skim milk solids	5	45	42	40	44	44	42	42 <sup>1,2</sup>
Beef blood solids	6	45	36	36	37	34	35	35 <sup>1,2</sup>

<sup>1</sup> The rats in this group were 180 days of age.

<sup>2</sup> Data are from one surviving rat.

Slightly lower values were usually found in the animals fed blood solids. The averaged erythrocyte data substantiate this general trend, as do the results obtained in the rats fed the various proteins at a 2.8% protein level, although there are considerable variations in the results in the latter instance. A general parallelism between the value of the various proteins for supporting somatic growth and for hemoglobin formation is suggested by these results.

The data obtained in the studies employing the hemorrhagic anemia technique, however, demonstrate more clearly a difference between the hemopoietic value of blood solids and the other proteins tested. It is evident from the average values given in table 4 that there is no significant difference between lactalbumin, casein, milk solids, and the mixture of beef blood and milk solids in their capacity to support hemoglobin regeneration after hemorrhage. The days after hemorrhage

TABLE 3  
*Group average hemoglobin and erythrocyte data.*

PROTEIN	NO. RATS	HEMOGLOBIN — GM/100 ML Days of age				ERYTHROCYTE COUNT AT 120 DAYS M/mm <sup>3</sup>
		26	77	90	120	
1. 18% Protein						
Lactalbumin	13	11.3	16.0	16.5	16.4	7.76
Casein	12	10.8	14.6	15.1	15.4	7.73
Blood solids-milk solids mixture	14	11.7	16.1	16.1	15.7	7.37
Skim milk solids	11	12.7	15.5	15.6	15.1	7.55
Beef blood solids	13	13.6	15.1	15.4	15.2	6.94
2. 2.8% Protein						
Lactalbumin	12	11.0	12.1	12.6	10.7	5.54
Casein	10	10.6	7.0	6.5		4.34 <sup>1</sup>
Blood solids-milk solids mixture	14	13.2	11.2	10.8	8.8	3.79
Skim milk solids	10	11.7	11.2	12.6	8.5	5.67
Beef blood solids	10	13.1	12.2	11.8	10.5	4.54

<sup>1</sup> Erythrocyte counts at approximately 77 days of age.

required for the hemoglobin level to regain the initial value and the mg hemoglobin regenerated per rat, per 100 gm body weight per day, and per gm dietary protein consumed per day are all similar. Paradoxically, however, in the rats fed beef blood solids as the source of protein (18% level) a consistently longer period was required for the hemoglobin level to return to normal and less hemoglobin was regenerated per rat per day, per 100 gm body weight per day, and per gm dietary protein consumed per day. The differences between the values

obtained in the animals fed blood solids and those fed the other proteins have been found to be statistically significant. Using the group fed casein for comparison, the following "significance ratios" were found: (1) mg hemoglobin per 100 gm body weight = 3.0; (2) mg hemoglobin per gm dietary protein = 10.3.

No consistent change in body weight of any of the animals occurred during the period of recovery from hemorrhage.

TABLE 4

*Effect of various proteins on hemoglobin formation in hemorrhagic anemia.*

PROTEIN	NO. RATS	AV BODY WT.	AV. PRO-TEIN DAILY	HR. <sup>-1</sup> DAYS TO INITIAL	MG HEMOGLOBIN FORMED PER DAY <sup>1</sup>		
					Per rat	Per 100 gm body wt	Per gm protein
<i>18% Level</i>		<i>gm</i>	<i>gm</i>				
Casein	10	531	2.1	15 ± 0.4	107.1 ± 7.1	20.0 ± 0.6	48.3 ± 3.0
Lactalbumin	12	523	2.0	17 ± 0.7	95.3 ± 4.5	18.1 ± 0.8	49.5 ± 1.9
Blood solids-milk solids mixture	12	477	1.9	16 ± 0.5	102.5 ± 4.4	21.9 ± 0.9	52.7 ± 2.1
Skim milk solids	7	358	1.7	15 ± 0.6	76.3 ± 5.7	21.2 ± 0.7	45.1 ± 3.3
Beef blood solids	10	93	1.1	21 ± 1.0	15.1 ± 1.3	16.4 ± 1.1	14.8 ± 1.2
<i>2.8% Level</i>							
Lactalbumin	6	88	0.13	28 ± 2.4	9.3 ± 1.7	10.0 ± 1.4	66.7 ± 6.8

<sup>1</sup> The values given are group averages with the probable errors of the means.

Of considerable interest also are the data obtained on the surviving rats fed lactalbumin at the low level (2.8% protein). As the averaged data show, a much longer time was required for the hemoglobin level to attain the initial value and much less hemoglobin was regenerated per rat per day and per 100 gm body weight per day. However, when calculated as mg hemoglobin regenerated per gm dietary protein consumed per day a strikingly different circumstance is seen—the mg hemoglobin regenerated per gm protein is greater than that formed when 18% protein as lactalbumin or other sources is provided. These differences have been found to be



statistically significant. Again using the group of rats fed 18% protein as casein for comparison, the following "significance ratios" were obtained: (1) mg hemoglobin regenerated per 100 gm body weight = 6.5; (2) mg hemoglobin per gm dietary protein ingested = 2.6. These observations are interpreted as further evidence that if the protein intake is restricted, hemoglobin formation has a "high priority" for the available protein in the organism and therefore hemoglobin synthesis takes precedence over the formation of general body tissue protein. This interpretation is in accord with that drawn from data obtained on dogs by Whipple and his associates (Whipple, '42).

#### DISCUSSION

The foregoing data emphasize the importance of both the quality and quantity of dietary protein for hemoglobin formation in the rat. The results indicate that, for the proteins studied, those proteins which are qualitatively best constituted for supporting somatic growth are also most effective for hemoglobin formation. Such a thesis is borne out by a variety of other evidence. If rats are fed a synthetic diet containing human or beef globin as the protein, poor growth occurs and an anemia develops (Orten, Bourque and Orten, '45). If the chief qualitative deficiency in the amino acid composition of globin is remedied, by the addition of isoleucine, an increased rate of growth occurs and the anemia is corrected. The same is true if the dietary intake of tryptophane (Albanese et al., '43) or lysine (Hogan et al., '41; Harris et al., '43) is inadequate.

The finding that the feeding of beef blood protein results in the maintenance of a somewhat lower hemoglobin level and slower rate of hemoglobin regeneration after hemorrhage than was found when lactalbumin or casein was fed was rather unexpected. It would appear probable that beef hemoglobin, the principle protein, should be superior since its amino acid composition should be similar to that of rat hemoglobin. Also, the work of Whipple and his associates (Miller,

Robscheit-Robbins and Whipple, '45) indicates an effective utilization of parenterally administered hemoglobin in forming new hemoglobin in the dog. The latter observations are complicated, however, by the fact that additional protein, or the required additional amino acids, could have been obtained from the dog's own tissue protein.

Two explanations of the paradoxical failure of beef blood protein to support satisfactory hemopoiesis in the rat seem possible. One, that rat hemoglobin differs widely in its amino acid composition from beef hemoglobin. Such a possibility seems rather unlikely, however, since although no data on the amino acid composition of rat hemoglobin are available, the values obtained on the hemoglobins of certain other species (Block, '34) indicate some general similarity rather than a wide dissimilarity.

Another, perhaps more logical explanation, is that hemoglobin is synthesized in the organism through some intermediate protein which contains, among other "essential" amino acids, isoleucine. Hence the feeding of the protein, hemoglobin, which is deficient in isoleucine, would result in an unsatisfactory rate of synthesis of the postulated intermediate protein and, in turn as was found, an unsatisfactory synthesis of hemoglobin. Such an hypothesis would also be in accord with the observations that other qualitatively deficient proteins are unsatisfactory for hemopoiesis and that proteins best suited for supporting satisfactory somatic growth are also best suited for hemoglobin formation.

The finding that, whereas dried beef blood is unsatisfactory for supporting somatic growth and hemoglobin formation, the mixture of dried beef blood and dried skim milk supported excellent body growth and hemopoiesis supports the thesis that the inadequacy of dried beef blood protein is due to a deficiency of one or perhaps more amino acids (Orten, Bourque and Orten, '45). Undoubtedly, the protein of dried skim milk supplies the principal lacking amino acid, isoleucine, in amounts sufficient to bring about a satisfactory rate of growth and hemopoiesis.

## SUMMARY

Five sources of protein have been studied with respect to their ability (1) to maintain normal hemoglobin levels in the growing rat and (2) to support hemoglobin regeneration after hemorrhage in the adult rat. The various preparations were fed to weanling animals at both 18% and 2.8% levels of protein for an experimental period of approximately 200 days.

At the 18% protein level, casein, lactalbumin, dried skim milk, and a mixture of dried skim milk and dried beef blood proved to be of about the same value, both for hemoglobin maintenance in the growing rat and for hemoglobin regeneration in the adult animal. On the other hand, dried beef blood protein was inferior, particularly for hemoglobin regeneration after hemorrhage.

There appeared to be a general parallelism between the hemopoietic value of the protein and its ability to support somatic growth.

At the 2.8% level of the various proteins, a mild to moderate anemia developed in all cases. However, only those animals receiving lactalbumin survived for the entire experimental period. In these animals, there was a significantly decreased rate of hemoglobin regeneration following hemorrhage. However, there was a very efficient utilization for hemoglobin regeneration of the limited amount of protein consumed.

These observations emphasize the importance of both the quality and quantity of dietary protein for hemopoiesis in the rat.

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